REMARKS

Claims 1-32 are pending in this application. Claims 9, 10, 18 and 19 have been withdrawn.

Claims 3-8, 11-17 and 20-32 are rejected under 35 U.S.C. §112, first paragraph, on the basis that the specification, although enabling for a method of reducing radiation resistance *in vitro* and *in vivo* through the administration of an antisense oligonucleotide that specifically targets and inhibits the expression of human HER-2, whereby human HER-2 expression is inhibited and radiation resistance is reduced, does not reasonably provide enablement for a method of reducing drug resistance *in vitro* or *in vivo* through the administration of an antisense oligonucleotide that specifically targets and inhibits the expression of human HER-2.

Applicants note with appreciation that the examiner has found the application is enabling for a method of decreasing radiation resistance in an organism through the administration of an antisense sequence that specifically targets human HER-2. The continued rejection of the claims as directed to the administration of such an antisense oligonucleotide to decrease drug resistance is traversed.

In maintaining this rejection, the examiner asserted that "the ability to treat radiation resistance is not predictive of the ability to treat cellular resistance to all drugs in an organism." Applicants respectfully submit that this is not correct. Enclosed herewith is a declaration by one of the inventors, Dr. Esther Chang. As Dr. Chang explains in her declaration, it is, in fact, well established in the literature, and thus well known to persons of ordinary skill in the art, that the same cell control pathways, with HER-2 at the top, can regulate <u>both</u> radiation and drug resistance. See, for example,

Schmidt and Lichtner, *Drug Resistance Updates* 5:11-18 (2002) and Grant et al., *Cancer Treatment and Research* 112:89-108 (2002), copies of which are provided with the declaration. These cell signaling pathways ultimately regulate programmed cell death (apoptosis). Down modulation of HER-2 in a drug/radiation resistant cell using SEQ ID NO:3 or other antisense HER-2 sequence changes the balance of these pathways towards cell death (i.e., sensitivity) rather than cell survival (i.e., resistance). Thus, when exposed to either radiation or chemotherapeutic agents, the cancer cells now respond by triggering the cell death pathway. Although different drugs and/or radiation may funnel into these cell control pathways differently, they all ultimately work through the same pathways leading to apoptosis. It is well known by those in the field that HER-2 is at the start of, and is a crucial modifier of, these various pathways (see Yardan and Slikowski, *Mol. Cell Biol.* 2:127-137 (2001), also provided) and that HER-2 levels can affect drug response (*Id.*; Alaoui-Jamali et al., *Biochemistry and Cell Biology* 75: 315-325 (1997); O'Gorman and Cotter, *Leukemia* 15:21-34 (2001), also provided). It should be noted that the same pathways indicated in Figure 6 of the present application also are provided in the above-cited references.

Data are provided in the examples set forth in the application which illustrate that the administration of a HER-2 antisense-containing complex in accordance with the present invention will treat drug resistance. Example 13 describes *in vitro* results obtained using four different human tumor cell lines: MDA-MB-435 (breast cancer), JSQ-3(head and neck cancer), DU145 (prostate) and U87 (glioblastoma). The data show these cell lines being sensitized to one of three different known chemotherapeutic agents (docetaxel, CDDP or gemcitabine) by

ligand-liposome delivered antisense HER-2. The example further provides the results of an *in vivo* experiment where MDA-MB-435 xenograft tumors were significantly sensitized to docetaxel (Taxotere®) in which tumor growth was inhibited by treatment with ligand-liposome-AS HER-2. See Figure 7 of the application. The application thus provides evidence of the usefulness of a HER-2 antisense to treat drug resistance in a host with cancer.

The examiner asserted that the "success of one antisense in targeting a particular target cell and target gene is not necessarily extrapolatable to the ability of another antisense directed to another target gene ... to successfully target the appropriate target cell or cells harboring the target gene of interest and successfully inhibit that target gene's expression whereby treatment effects are provided" in the host. Applicants wish to point out to the examiner that, as stressed in Dr. Chang's declaration, they are claiming only the use of an antisense oligo that targets HER-2, whether over-expressed or not. They are not extrapolating to another antisense molecule which binds to a different target gene as the examiner asserts. As HER-2 is at the top of the signaling pathways, down-modulating HER-2 affects signaling throughout the pathways which involve other genes, such as those listed in claims 8 and 17 of the present application. It must be emphasized that Applicants have not asserted the use of an antisense molecule against any of these genes, simply that abnormalities in these genes can result in chemo-resistance as well as radiation resistance. In view of HER-2's location in the pathway involving these genes, an antisense to HER-2 affects the entire pathway and so can sensitize the cells to these therapies.

All of the pending claims focus on the use of an antisense oligo to the HER-2 gene. As noted above, down modulation of HER-

2 can influence and reduce drug resistance through the pathways initiated by HER-2 even where HER-2 is not over-expressed, as in the MDA-MB-435 (breast), DU145 (prostate), JSQ-3 (head and neck) and U 87 (glioblastoma) tumor cell lines. *In vitro* and *in vivo* illustrations of this also are provided in the attached declaration by Dr. Chang. The declaration describes studies involving pancreatic cancer cell lines PANC-1 and/or COLO 357, which express high and low levels of HER-2, respectively. Each carries a mutated, active form of the *ras* gene, an important component of the RAF-MAPK signaling pathway (See Figure 6 of the application). *In vitro* treatment of both of these cell lines with ligand-liposome-AS HER-2 resulted in a significant (i.e. greater than 12 fold) decrease in drug resistance. More importantly, when PANC-1 (low HER-2 expressor) xenograft tumors were treated with AS HER-2 delivered intravenously by the ligand-liposome complex, they were more responsive (sensitive) to the first line chemotherapeutic agent Gemzar (gemcitabine) and showed tremendous growth inhibition compared to those receiving Gemzar alone. These data illustrate that antisense HER-2 can reverse drug resistance where another gene in the signal transduction pathway (in this instance, ras) is abnormal, whether HER-2 is over-expressed or not.

In view of the foregoing discussion and data, Applicants respectfully submit that they have shown that the application is enabling for the treatment of drug resistance as well as radiation resistance. The method of treatment is effective for both drug and radiation resistance.

Claims 1 and 2 have been rejected under 35 U.S.C. §102(b) as anticipated by Adams et al., *Nature* 377 (supp):3-17 (1995). The examiner asserted that the reference teaches an isolated compound comprising SEQ ID NO:3 ("see the accompanying sequence alignment

data between SEQ ID NO:3 and accession No. AA360512"). This rejection is traversed.

Applicants respectfully submit that the cited reference does not teach or even suggest the therapeutic agent of claims 1 and 2 of the present application.

As an initial point, Applicants wish to point out that the Accession number referenced by the examiner, Accession No. AA360512, is the 5' end of a short piece of a cDNA sequence in the "Hereditary Multiple Exostosis Gene 2" derived from a human T cell lymphoma. A sequence comparable to that of SEQ ID NO:3 is near the 3' end of this short piece of a sense DNA strand. SEQ ID NO:3, however, is an antisense sequence designed to bind to the sense strand of HER-2 RNA near its initiation codon. The AA3605512 sequence thus has absolutely no relationship to SEQ ID NO:3 or to antisense HER-2.

Furthermore, none of the sequence of SEQ ID NO:3, HER-2 or accession number AA360512 (or its gene) is even mentioned in the cited reference. Ths paper describes only the methods used to obtain, and the "preliminary characterization" of (as part of the human genome project) more than 87,000 of these expressed sequences tags (ESTs) with regard to the tissues in which they are expressed. There is no identification of the sequence identified herein as SEQ ID NO:3, and certainly no function or use for any of th ESTs is described or even postulated. In particular, no mention is made of the down-modulation of genes in a cell signaling pathway in a cancer cell. No suggestion is made as to how these ESTs might be used, if indeed that is ever possible, for cancer therapy.

In view of the foregoing amendments and discussion, Applicants respectfully submit that the pending claims are in condition for allowance.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	*Application Number*	09/716,320
	Filing Date	
	First Named Inventor	Esther H. Chang
	Group Art Unit	1635
	Examiner Name	
	Attorney Docket Number	2444-109
Title of the Invention:		

DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Esther Chang, declare that:

1. I am the same Esther Chang named as an inventor on the above-referenced patent application.

2. I received a B.A. degree in biology from Fu Jen University in Taiwan in 1968 and a Ph.D. in microbiology from Southern Illinois University in 1974. From 1982-1994 I held the positions of Assistant Professor, Associate Professor, and then Professor in the Department of Pathology, Uniformed Services University of the Health Sciences in Bethesda, MD. I also was a Research Professor in their Department of Surgery and the Director of their Tumor Biology Program. From 1994-1996 I held the position of Professor of Surgery (Research), Division of Otolaryngology/Head and Neck Surgery in the Department of Surgery

at Stanford University Medical Center. Since 1996, I have held the position of Professor of Surgery (Consultant) there. I currently also hold the positions of Professor of Otolaryngology, Department of Otolaryngology/Head & Neck Surgery and Professor of Oncology and Otolaryngology, Departments of Oncology and Otolaryngology, at the Georgetown University Medical Center, Lombardi Cancer Center, and have held those positions since 1996 and 1999, respectively. A copy of my curriculum vitae is attached hereto.

3. I have read the Office Action issued by the U.S. Patent and Trademark Office on September 23, 2003, and understand the grounds of rejection set forth therein.

4. In one rejection the examiner has asserted that claims 3-8, 11-17 and 20-32 are not fully enabled on the basis that, although the specification is enabling for a method of reducing radiation resistance *in vitro* or *in vivo* through the administration of an antisense oligonucleotide that specifically targets and inhibits the expression of human HER-2, the specification does not enable a method of reducing drug resistance *in vitro* or *in vivo* through the administration of such an antisense oligonucleotide. More specifically, the examiner asserted that the ability to treat radiation resistance is not predictive of the ability to treat cellular resistance to drugs in

an organism (host). She stated that undue experimentation would be required to determine the efficacy of an antisense in successfully targeting and inhibiting a target gene in an organism and that the *in vivo* efficacy of antisense in target gene inhibition and providing treatment effects has to be derived empirically for the particular gene in the organism and for determining treatment effects provided by antisense administration.

5. Contrary to the examiner's assertion that the ability to treat radiation resistance in a host is not predictive of the ability to treat drug resistance, it is well-established in the literature that the same cell control pathways, with HER-2 at the top, regulate both radiation and drug resistance. See, for example, Schmidt and Lichtner, *Drug Resistance Updates* 5:11-18 (2002), which provides that the signaling pathways that involve the EGFR family (e.g., HER-2; see Figure 1) influence chemoresponse, and discusses "mechanisms by which EGFR inhibition sensitizes tumor cells to chemo- or radiation therapy" (page 13), and Grant et al., *Cancer Treatment and Research,* pp. 89-108 (2002), which, as the title of the chapter provides, focuses on the "role of signal transduction pathways in drug and radiation resistance." Both references are attached hereto. It is known

that these cell signaling pathways ultimately regulate programmed cell death, i.e., apoptosis. By down-modulating HER-2 in a drug or radiation resistant cell using an antisense HER-2 sequence, such as SEQ ID NO:3, the balance of these pathways towards cell death (i.e., sensitivity), rather than survival (i.e., resistance), changes. As a result, when exposed to either radiation or a chemotherapeutic agent, the cancer cells now respond by triggering the cell death pathway. Although different drugs and/or radiation may funnel into the cell control pathways differently, they all ultimately work through the same pathways leading to apoptosis. It is well known to those of skill in the art that HER-2 is at the start, and is a crucial modifier, of these various pathways. See Yardan and Slikowski, *Mol. Cell Biol.* 2:127-137 (2001), attached hereto, a review paper which points out that HER-2 (erbB2) is at the top of the multiple signal transduction pathways that are involved in drug resistance. It is well known to persons of skill in the art that HER-2 levels can affect drug response. *Id.* See also Alaoui-Jamali et al., *Biochemistry and Cell Biology* 75:315-325 (1997), which notes in the abstract that "of particular interest is the intrinsic drug resistance associated with over-expression of the erbB-2 (HER-2) receptor" and that "the apoptotic signal induced by many anticancer drugs originates at a receptor on the cell membrane and

is transduced through a signaling cascade to the nucleus," and O'Gorman and Cotter, *Leukemia* 15:21-34 (2001), which provides that it now is "widely accepted that chemotherapeutic drugs kill tumor cells by inducing apoptosis" and that the "exploitation of survival pathways ... may also be important in the development of chemoresistance." This latter paper discusses the association between multiple signaling cascades and their contribution to the development of the chemoresistant phenotype. Both of these papers also are attached hereto.

6. In explaining the rejection under 35 U.S.C. §112, first paragraph, the examiner also stated that one cannot necessarily extrapolate from the success of one antisense oligonucleotide in targeting a particular target cell and target gene to the ability of another antisense oligo directed to another target gene to successfully target the appropriate cell or cells harboring that target gene of interest and inhibit the expression of that target gene. This statement simply is not relevant to the presently claimed invention, as we are claiming <u>only</u> the use of an antisense molecule which targets HER-2, whether over-expressed or not. There is no discussion of, and no extrapolation to, another antisense molecule to a different target gene. HER-2 is at the top, or initiation point, of the signaling pathways, which means that down-modulating HER-2 will affect signaling <u>throughout</u> the

pathways. These pathways, in turn, involve other genes, such as those listed in claims 8 and 17. That is, the genes are involved in HER-2 dependent cell control pathways and can be abnormally expressed in addition to, or in place of, abnormal expression of HER-2.

We are not claiming the use of an antisense molecule against any of these other genes, however; rather, our invention is taking advantage of two facts: (1) abnormalities in these genes can result in chemo or radiation resistance and (2) the location of HER-2 in the pathway involving these genes means that the down-modulation of HER-2 will affect subsequent parts of the pathway. As the administration of an antisense oligonucleotide to HER-2 affects the entire pathway involving these genes, it can sensitize the cells to the desired therapy.

7. As I have noted, down modulation of HER-2 can influence and reduce drug or radiation resistance through HER-2 dependent cell control pathways even if HER-2 is not over-expressed, such as in MDA-MB-435, DU145, JSQ-3 and U87 tumor cell lines. *In vitro* and *in vivo* tests illustrating the effectiveness of an antisense oligo to HER-2 with these cell lines is shown in Example 13 of our patent application. A further illustration is described below. The experimental work described below was carried out in my laboratory under my direction and instructions.

Pancreatic cancer cell lines PANC-1 and COLO 357 were used in these tests. The two cell lines express, respectively, low and high levels of HER-2. Each also carries a mutated, active form of the *ras* gene, an important component of the RAF-MAPK signaling pathway (see Figure 6 of our application). *In vitro* treatment of both of these cell lines with a ligand-liposome-antisense HER-2 complex resulted in a significant (greater than 12 fold) decrease in drug resistance. Furthermore, when PANC-1 xenograft tumors were treated with antisense HER-2, delivered intravenously by means of a ligand-liposome complex of our application, they were more responsive (sensitive) to the known first line chemotherapeutic agent Gemzar® (gemcitabine) and showed tremendous growth inhibition in comparison to such xenografts receiving Gemzar® alone. These data demonstrate that antisense HER-2 can reverse drug resistance when a gene in the signal transduction pathway is abnormal, regardless of whether HER-2 is over-expressed.

These experiments are described in detail in Attachment A to this declaration.

8. I further declare that all statements made herein and in Attachment A of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.

Esther Chang, Ph.D.

1/22/04
Date

Attachment to Declaration
U.S. Serial No. 09/716,320

ATTACHMENT A

In vitro Chemosensitization

MDA-MB-435, PANC-1 and COLO 357 cells (5 x 10^3 cells/well for MDA-MB-435 and 4 x 10^3 cells/well for PANC-1 and COLO 357) in 100 µl of the appropriate medium were plated in triplicates in a 96 well microtiter plate. Following overnight incubation, the cells were washed twice with serum free medium, overlaid with 50 µl of a transfection complex containing the antisense HER-2 oligonucleotide in serum free medium and incubated for 12 hours. For the MDA-MB-435 cells the complex was folate-liposome 2-AS HER 2 at a ratio of folate:liposome of 2 µmol/ml total lipid and folate-liposome:antisense oligo of 20 nmol:2 nmol. Liposome 2 comprises a 1:1 molar ratio of dimethyl dioctadecylammonium bromide:dioleoylphosphatidylethanolamine (DDAB:DOPE). For PANC-1 and COLO 357 cells the complex was TfRscFv-LipA-AS HER2 at a ratio of TfRscFv:liposome of 1:30 (wt:wt) and antisense oligo:liposome of 1:15 (nmol:nmol). In this complex, the ligand is a single chain antibody fragment which binds to the transferrin receptor and the liposome comprises a 1:1 molar ratio of dioleoyltrimethylammonium-propane:dioleoylphosphatidylethanolamine (DOTAP:DOPE). Fifty µl of the appropriate medium, supplemented with 20% FCS, 4 mM L-glutamine and antibiotics then were added. The cells then were incubated for an additional 12 hours, followed by addition of 100 µl of the appropriate supplemented medium, with or without a chemotherapeutic drug, and incubation continued for approximately 72 hours. The chemotherapeutic drugs used were Taxol® (paclitaxel), Taxotere® (docetaxel) and Gemzar®

(gemcitabine). After incubation for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2, the wells were washed once with phosphate buffered saline (PBS) and the cell viability XTT-based assay was performed according to the manufacturer's protocol (Boehringer Mannheim). In the presence of an electron-coupling reagent, XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonate) is converted into orange formazan by dehydrogenase in the mitochondria of living cells. The formazan absorbance that correlates to the number of living cells was measured at 450 nm using a microplate reader. The IC_{50} was interpolated from the graph of the log of drug concentration vs the fraction of surviving cells.

Drug Sensitization of Low HER-2 Expressing Breast Cancer Cells by Antisense-HER-2 Oligonucleotide

Taxotere®, like Taxol®, is a member of the taxane family. Taxotere is one of the most active single chemotherapeutic agents for the treatment of metastatic breast cancer and is now standard therapy in clinical practice. Although Taxol® and Taxotere® have a similar mechanism of action, they are significantly different in clinical characteristics related to their efficacy/toxicity ratio relative to dose and schedule.

The level of sensitization to both taxane-based drugs was examined. The antisense HER-2 oligonucleotide (AS-HER-2 ODN) employed in these studies is that identified in the patent application as SEQ ID NO:3, a phosphorothioate pentadecamer complementary to the initiation codon region of HER-2 mRNA. AS-HER-2 concentrations of 0.1 μM and 0.3 μM were tested. These two concentrations were chosen as they both were below the IC_{50} value (i.e., the concentration of treatment agent resulting in a 50%

decrease in viable cells) for AS-HER-2 alone in the breast cancer cell line MDA-MB-435. This is important as high concentrations of AS-HER-2 itself can inhibit cell growth. We also had observed previously that for MDA-MB-435 cells, AS-HER-2 concentrations greater than 0.3 μM, when used in combination with a chemotherapeutic agent, resulted in such a high degree of cell death that an accurate determination of IC_{50} was not possible. In the assays evaluating sensitization of MDA-MB-435 cells by a complex of folate-liposome-2-AS-HER-2, MDA-MB-435FF cells (i.e., cells adapted for growth in a folate-free medium) were used so that the high levels of folate in the medium would not inhibit the binding of the folate ligand in the complex to the cellular receptor to demonstrate proof-of-principle. It should be noted, however, that in the *in vivo* studies discussed below, the same F-Lip-2-AS-HER-2 ODN complex is administered through the blood stream.

The degree of sensitization to the drug is based upon comparison of the IC_{50} values after transfection with AS HER-2 ODN vs. transfection with scrambled (SC)-HER-2 ODN (Fold sensitization = IC_{50} SC/IC_{50} AS). (The scrambled antisense has the same nucleotide composition as the antisense oligo of interest but in random order and is used as a negative control to demonstrate that the effect obtained is antisense specific.) The results are given in Table 1 and representative survival curves are shown in Figures 1A, 1B and 1c.

AS-HER-2 ODN delivery mediated by folate-liposome-2 targeted delivery system resulted in a 6.7 and 7.2 fold (Figure 1A) increase in cell killing by Taxol® and Taxotere®, respectively, at an antisense concentration of 0.1 μM. At an AS-HER-2 ODN concentration of 0.3 μM, the amount of sensitization increases

significantly (Figures 1B and 1C), particularly with Taxotere®, where there was a 30 fold increase in response of the cells to the chemotherapeutic agent after treatment with the folate-liposome-2-AS-HER-2 complex (Figure 1C). (Figure 1C is the survival curve for the results described in Example 13 of the application for MDA-MB-435 cells).

One important point to note is that the range of AS-HER-2 ODN (0.1 - 0.3 μM) used in these studies to demonstrate sensitization to chemotherapeutic agents is the same range previously given in the application for sensitization to radiation.

In Vitro Drug Sensitization of Low and High HER-2 Expressing Pancreatic Cancer Cells that Carry a Mutated Form of the *ras* Gene by AS-HER-2 ODN

The anti-metabolite Gemzar® is the most recent drug to be employed as a first-line treatment of pancreatic cancer (PanCa). Although numerous studies have indicated that Gemzar® has greater clinical benefit than the most widely used alternative, 5-FU, the improvement in quality of life and survival is only modest, and its primary effects are palliative. It would be a significant advance in the use of this drug for treating PanCa patients if the tumor targeted liposome-AS-HER-2 complex could result in an increased response to this agent through the down modulation of HER-2. The XTT cytotoxicity assay described above was used in these studies to establish the level of chemosensitivity induced by a complex comprising an anti-transferrin receptor single chain antibody fragment (TfRscFv) as the ligand, or targeting moiety (like transferrin itself, this molecule targets and binds to the

transferrin receptor (TfR)), a liposome comprising DOTAP:DOPE in a 1:1 molar ratio (referred to herein as liposome A or LipA) and the antisense HER-2 oligo identified in the application as SEQ ID NO:3. Two days after transfection, Gemzar® was added at increasing concentrations (in triplicate). The XTT assay was performed approximately 4 days later and the IC_{50} values, the drug concentration yielding 50% growth inhibition, was calculated. Significant chemosensitzation was observed. Treatment with the TfRscFv-LipA-AS HER-2 complex (at an oligonucleotide concentration of 1μM) increased the response of PANC-1 cells (a low HER-2 expresser) to Gemzar® by over 12 fold (see Figure 2A). A similar level of chemosensitization was observed with COLO 357 cells (high HER-2 expressor) but at a much lower concentration of AS-HER-2 (0.25μM) (Figure 2B). In both cases, treatment with the complex carrying the SC ODN had minimal effect. Although COLO 357 is inherently more responsive to Gemzar® than PANC-1, as is evident by comparing the IC_{50} value of the two cell lines, 0.58 nM vs. 560 nM), treatment with the AS HER-2 complex is still capable of significant chemosensitization of COLO 357. Both cell lines carry mutant, activated versions of the *ras* oncogene. In Figure 2A, the efficiency of sensitization of the complex targeted by the TfRscFv was compared to that of Tf itself. The survival curve and IC_{50} values of both complexes are virtually identical, indicating that the TfRscFv binds to the Tf receptor as efficiently as Tf itself. Therefore, these *in vivo* studies demonstrate that this gene delivery system has the potential to effectively treat PanCa.

In Vivo Chemosensitization of Pancreatic Cancer Tumors by TfRscFv-LipA-AS-HER-2

The *in vitro* studies described above indicated that

treatment of pancreatic cancer cells with the AS-HER-2 complex could increase their response to Gemzar®. For this gene therapy delivery system to be clinically relevant for pancreatic cancer, the increased sensitization observed *in vitro* must translate to an *in vivo* model. For proof-of-principle, the efficacy of the TfRscFv-LipA-AS HER-2 in treating pancreatic cancer was assessed using the subcutaneous PANC-1 tumor xenograft mouse model. Athymic nude mice (5-9 mice/group with two tumors/mouse) bearing subcutaneous xenograft tumors of ~50 mm^3 were treated three times per week with the TfRscFv-LipA-AS HER-2 complex containing ODN at 9 mg/kg/injection (~36 nmoles/mouse/injection). As controls, one group of animals received Gemzar® alone, the TfRscFv-LipA-AS HER-2 alone, or the combination of Gemzar® and the complex carrying the single chain oligonucleotide. Gemzar® was given I.P. twice weekly at 60 mg/kg. The animals received a total of 18 i.v. injections of complex and 12 of Gemzar®. As shown in Figure 3, Gemzar® alone had only minimal effect on tumor growth, while the AS HER-2 alone was ineffective. The groups receiving Gemzar® alone or control SC ODN plus Gemzar® are not statistically different, indicating that any growth inhibition by TfRscFv-LipA-SC ODN was strictly a drug effect. Tumor growth was substantially inhibited, however, in the mice who received the combination of AS HER-2 in the complex with the ligand-liposome, delivered via intravenous injection, and the chemotherapeutic agent Gemzar®. The differences between the group receiving the combination therapy and Gemzar® alone or TfRscFv-LipA-AS HER-2 alone are highly statistically significant ($P<0.001$ by student's t-test). Thus, the i.v. administration of the complex carrying the AS HER-2 can sensitize PanCa tumors to the standard chemotherapeutic agent Gemzar® and is efficacious against PanCa.

We also monitored the weights of the animals as an indicator of toxicity. No weight loss occurred and there was no significant difference between any of the treatment groups. Thus, the TfRscFv-LipA- AS HER-2 has no major non-specific cytotoxicity. Therefore, this study strongly indicates that the systemically delivered, tumor targeted liposome-AS HER-2 complex can sensitize pancreatic cancer tumors to chemotherapeutic agents resulting in more effective treatment.

Attachment to Declaration
U.S. Serial No. 09/716,320

Figure Legends

Figures 1A-1C: *In vitro Sensitization of MDA-MB-435 cells to Taxol® and Taxotere® by Folate-Lip2-AS-HER-2 ODN*

XTT assays were performed to assess the degree of sensitization to either Taxol® or Taxotere® after transfection with F-lip2-AS-HER-2-ODN. Each point is the mean of triplicate samples ± standard deviation. F = folate; Lip2 = a liposome comprising 1:1 DDAB:DOPE; AS = antisense HER-2 ODN; SC = scrambled HER-2 ODN; Fold Sensitization = IC_{50} SC/IC_{50} AS.

Figure 1A: Taxol® sensitization with F-Lip2 at an AS HER-2 ODN concentration of 0.3 μM.

Figure 1B: Taxotere® sensitization with F-Lip2 at an AS HER-2 ODN concentration of 0.1 μM.

Figure 1C: Taxotere® sensitization with F-Lip2 at an AS HER-2 ODN concentration of 0.3 μM.

Figures 2A and 2B: *In vitro Chemosensitization of PANC-1 and Colo 357 PanCa cell to Gemzar® by TfRscFv-LipA-AS HER-2*

The cells were seeded in a 96 well plate and 24 hours later transfected with TfRscFv-LipA complex containing 0.25 or 1 μM of either AS HER-2 or SC ODNs for 24 hours followed by addition of Gemzar®. As controls, untransfected cells and cells treated with either TfRscFv-LipA (LipA) alone or the complex carrying Tf as the targeting ligand were used. The XTT assay was performed following a 72 hours incubation with the drug. In some cases the error bars are too small to be visualized on the graph. Panel A =

sensitization of PANC 1 cells; Panel B = sensitization of Colo 357 cells.

Figure 3: *In vivo Effect of the Combination of TfRscFv-LipA-AS-HER-2 and Gemzar® (gemcitabine) treatment on PANC-1 Xenograft Tumors*

Athymic nude mice carrying human PANC-1 subcutaneous xenograft tumors were injected i.v. with TfRscFv-LipA-AS HER-2 alone and in combination with Gemzar® (60mg/kg). 18 injections of complex and 12 injections of Gemzar® were given. AS and SC ODN concentrations are 9 mg/kg. Day 0 represents the tumor volume prior to initiation of treatment. Values are given as mean tumor volume (mm^3) ± S.E. of 10-18 tumors/group.

Table 1

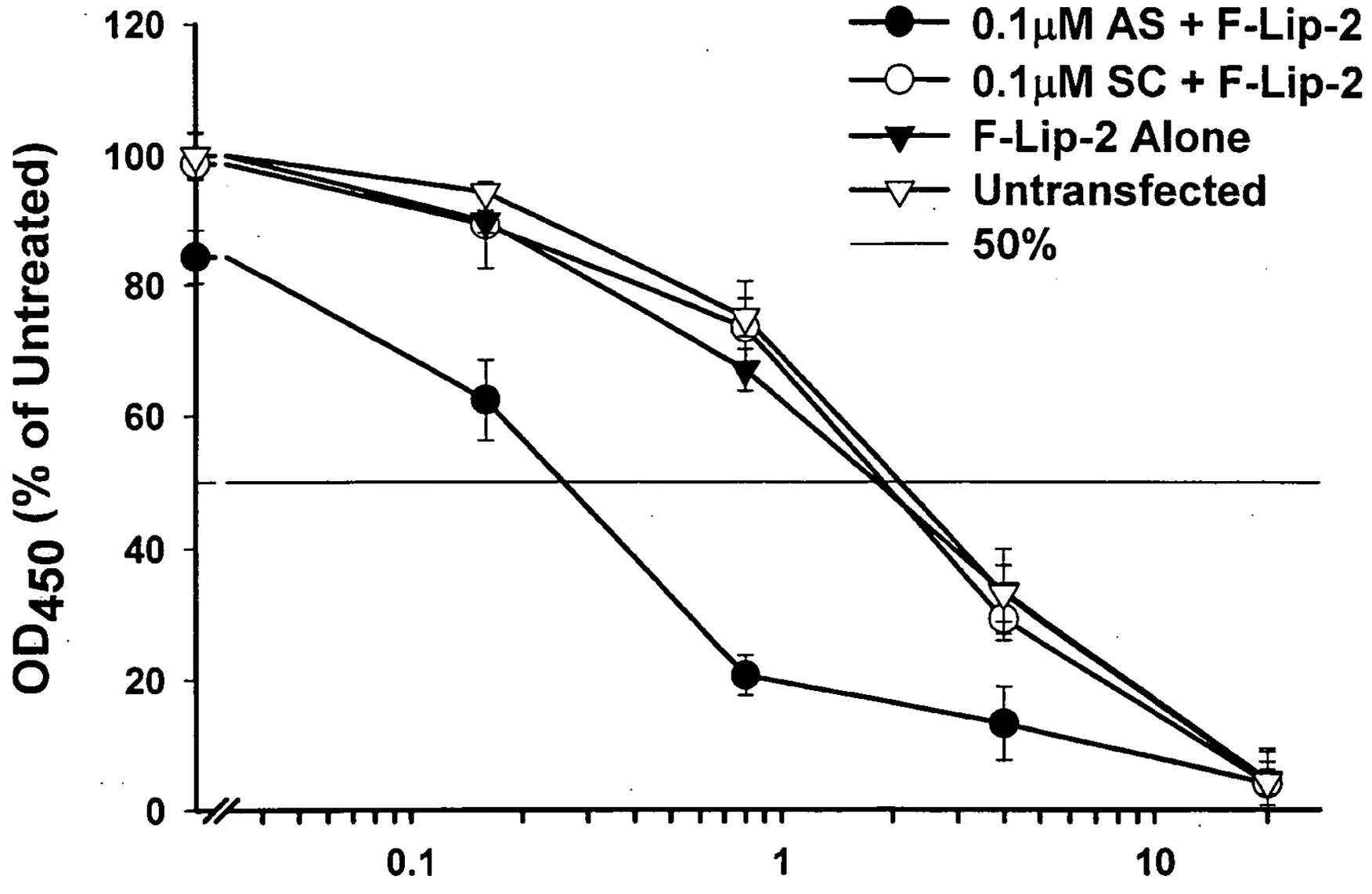
Chemosensitization of Human Breast Cancer MDA-MB-435 cells by AS-HER-2 ODN

Delivery Vector:	F-Lip-2	
AS HER-2 ODN Concentration:	0.1μM	0.3μM
Fold Sensitization* to Taxol:	6.7	9
Fold Sensitization* to Taxotere:	7.2	>30

*Fold Sensitization = Ratio of IC_{50} for SC HER-2 ODN to IC_{50} for AS HER-2 ODN

FIGURE 1A

Chemosensitization of MDA-MB-435 Cells by F-Lip-2- AS-HER-2 ODN



IC_{50}:

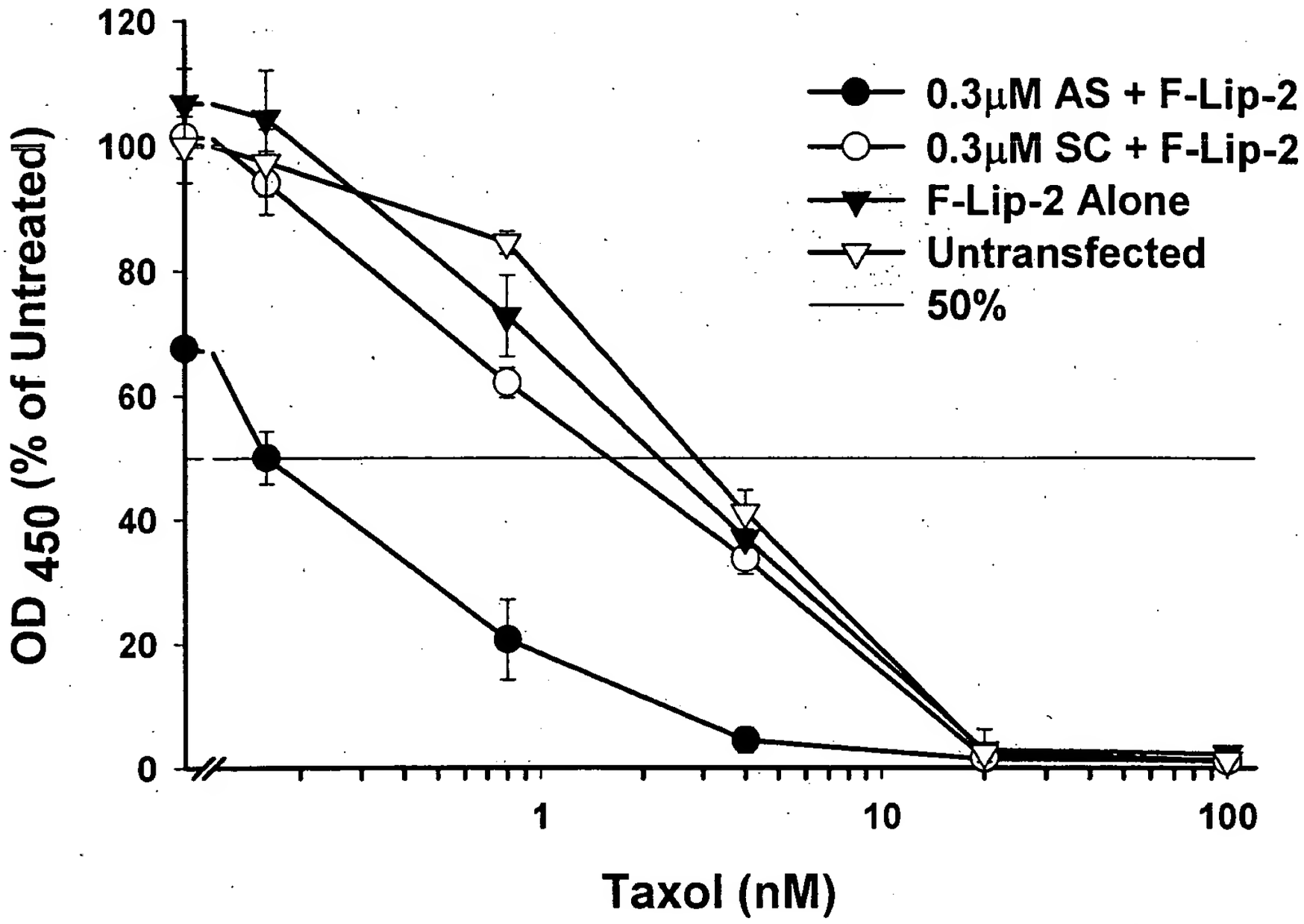
0.1μM AS + F-Lip-2	0.25nM
0.1μM SC + F-Lip-2	1.8nM
F-Lip-2 Alone	2.1nM
Untransfected	2.3nM

Fold Sensitization:

IC_{50} SC / IC_{50} AS = 7.2

FIGURE !B

Chemosensitization of MDA-MB-435 Cells by F-Lip-2- AS-HER-2 ODN



IC_{50}:

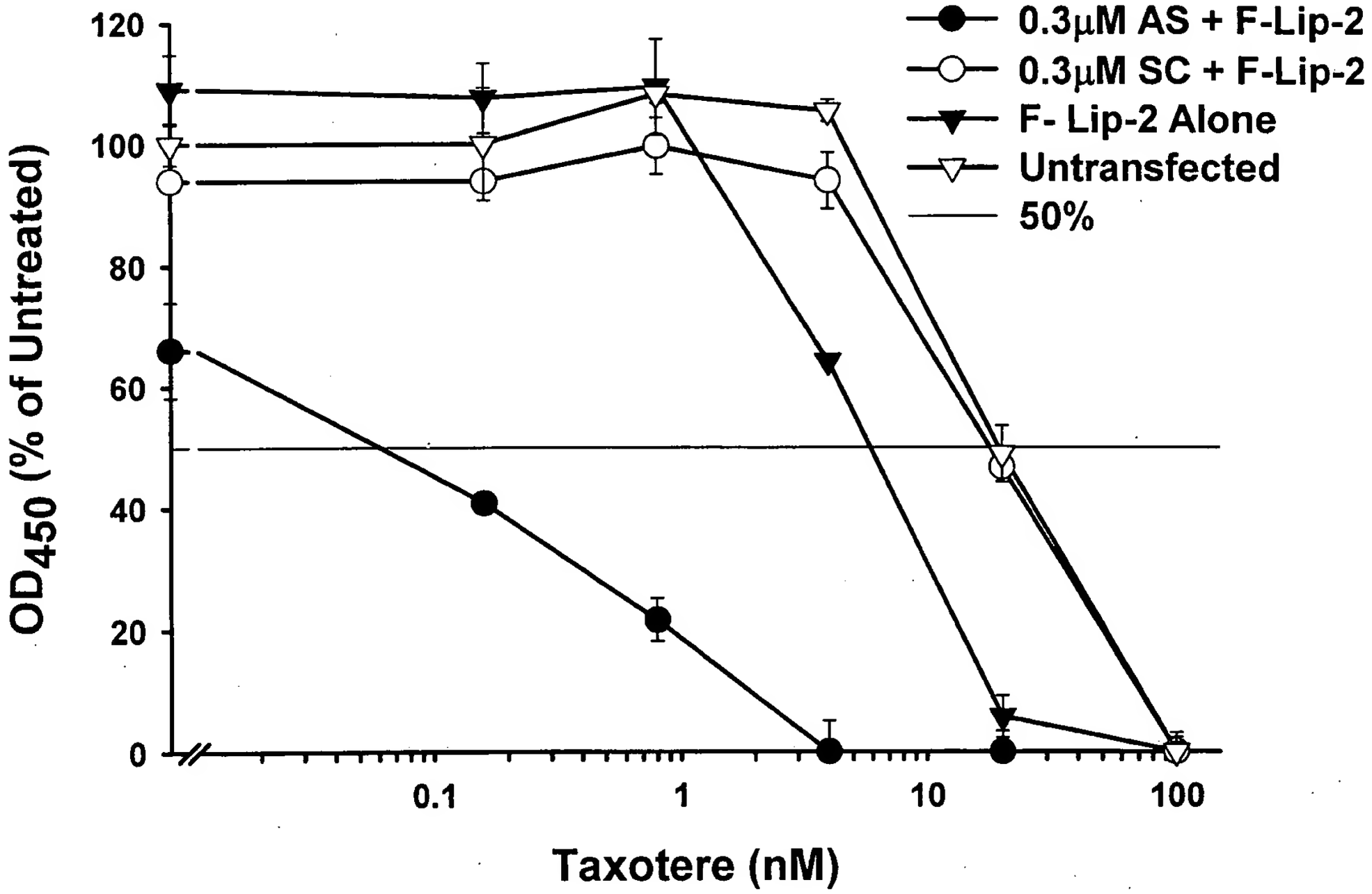
0.3μM AS + F-Lip-2	0.15nM
0.3μM SC + F-Lip-2	1.4nM
F-Lip-2 Alone	2.1nM
Untransfected	3nM

Fold Sensitization:

IC_{50} SC / IC_{50} AS = 9

FIGURE 1C

Chemosensitization of MDA-MB-435 Cells by F-Lip-2-AS-HER-2 ODN



IC_{50}:

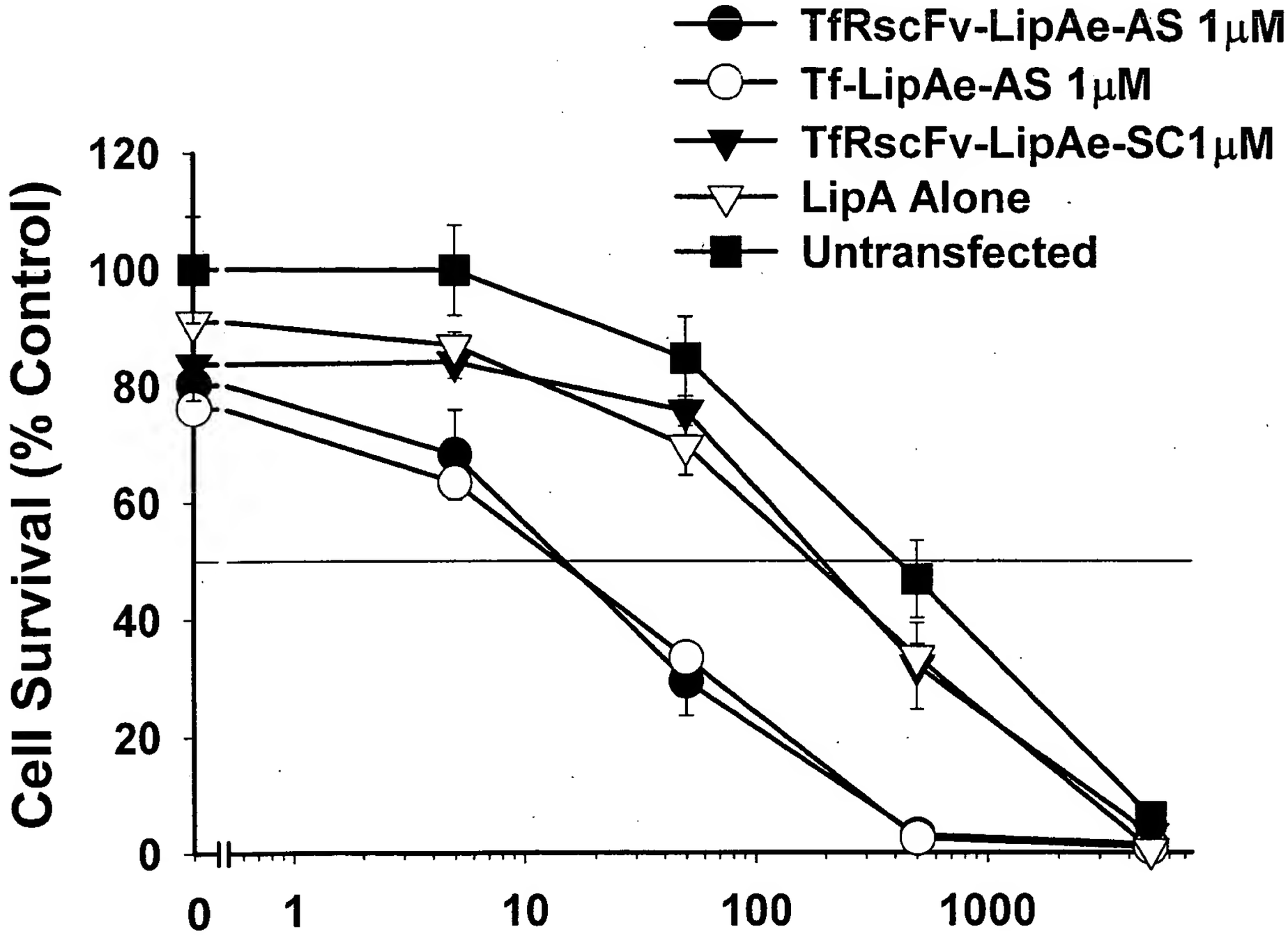
0.3μM AS + F-Lip-2	0.2nM
0.3μM SC + F-Lip-2	6.1nM
F-Lip-2 Alone	17.2nM
Untransfected	20nM

Fold sensitization:

$IC_{50}SC / IC_{50} AS = 30.5$

FIGURE 2A

TfRscFv-LipAe-AS-HER-2 ODN Mediated Sensitization of Panc-1 Cells to Gemzar



IC_{50}

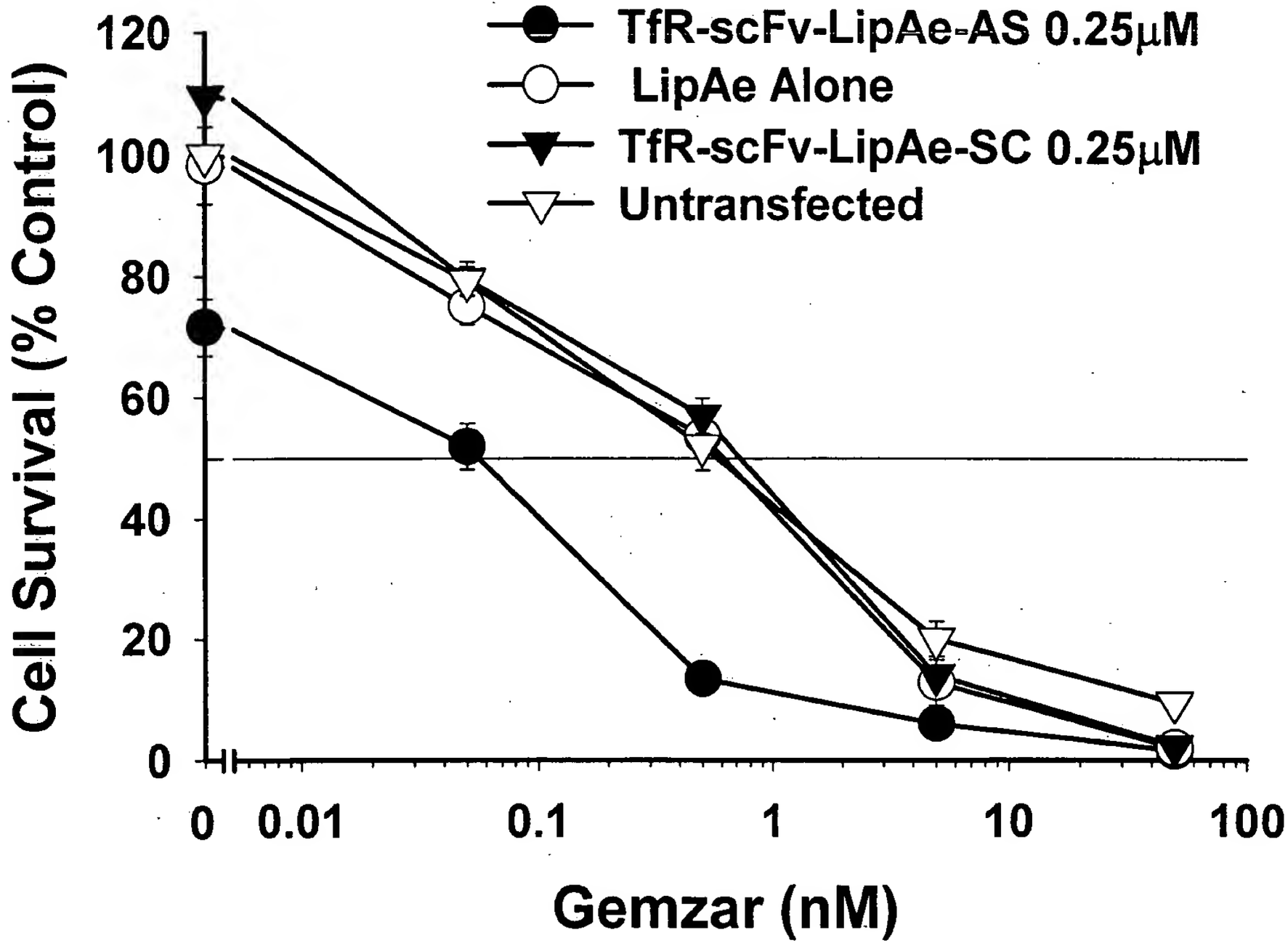
TfR-scFv-LipAe-AS	16nM
Tf-LipAe-AS	14nM
TfR-scFv-LipAe-SC	200nM
Untransfected	560nM

Fold Sensitization:

IC_{50} SC / IC_{50} AS = 12.5

FIGURE 2B

TfR-scFv-LipAe-AS-HER-2 ODN Mediated Sensitization of Colo 357 Cancer Cells to Gemzar



IC_{50}

TfR-scFv-LipAe-AS-HER-2	0.06nM
Lip Alone	0.61nM
TfR-scFv-LipAe-SC-HER-2	0.75nM
Untransfected	0.58nM

Fold Sensitization:

IC_{50} SC / IC_{50} AS = 12.5

PANC I Xenograft Tumors I.V. Treated with the Combination of TfRscFv-LipA-AS HER-2 Plus Gemzar

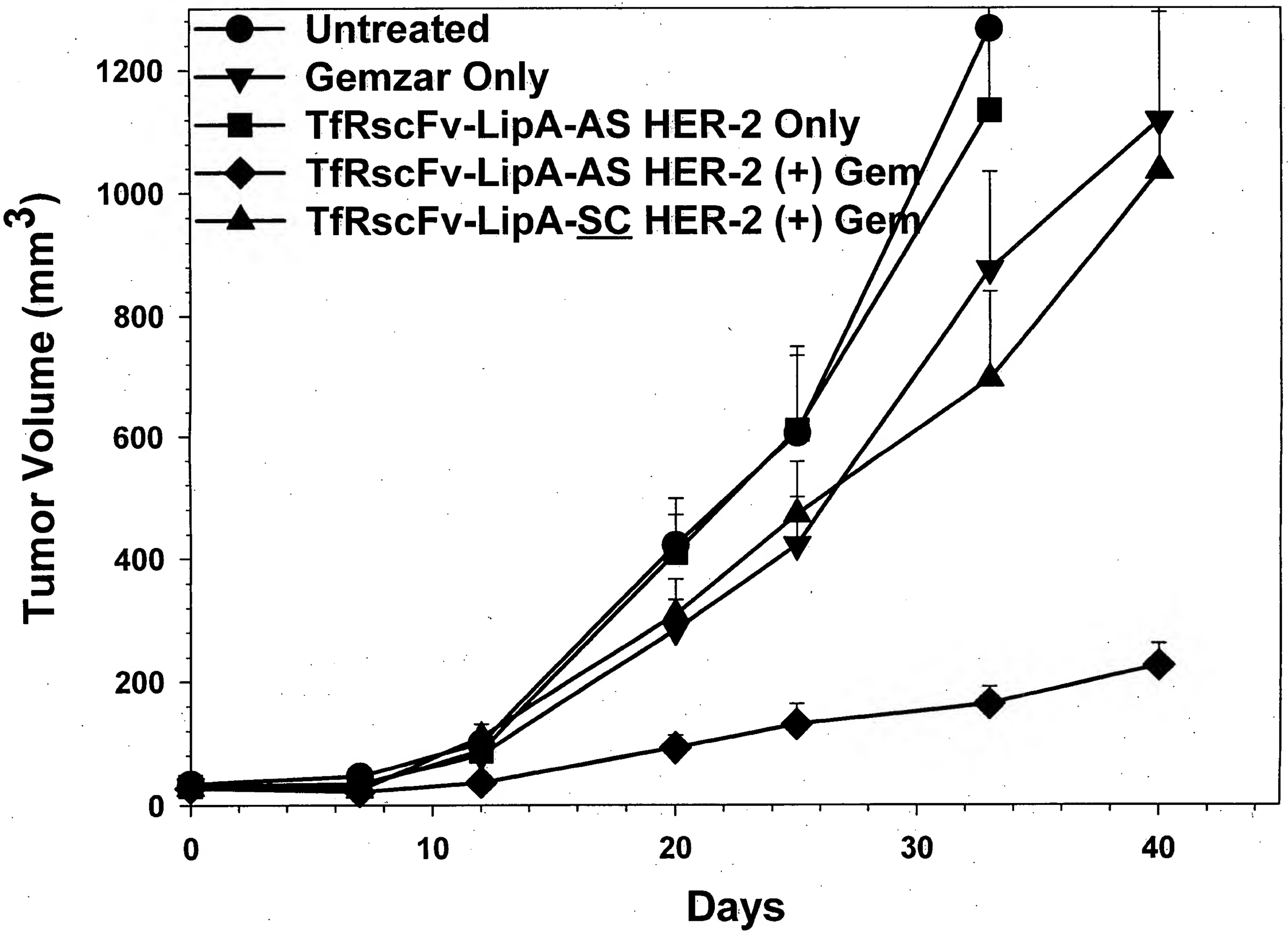


FIGURE 3

CURRICULUM VITAE

10/7/2002

PERSONAL

Name: Esther H. Chang
Place of Birth: Chungking, China
Citizenship: U.S. Citizen
Marital Status: Married with 1 daughter (Harford)
Work Address: Departments of Oncology & Otolaryngology
Georgetown University Medical Center
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EDUCATION

Fu Jen University, Taiwan	B.A.	1968	Biology
Southern Illinois University	Ph.D.	1974	Microbiology

PROFESSIONAL APPOINTMENTS

Trainee
U.S. Naval Medical Research Unit No. 2
Taiwan
1967 - 1968

Research Assistant
Southern Illinois University
1968 - 1971

Teaching Assistant in Immunology and Virology
Southern Illinois University
1971 - 1972

Research Associate
Southern Illinois University
1972 - 1973

Special Dissertation Fellow
Southern Illinois University
1973 - 1974

Visiting Fellow
National Institutes of Health
1974 - 1977

Visiting Associate
National Institutes of Health
1977 - 1978

Cancer Expert
National Cancer Institute
1978 - 1982

Assistant Professor
Department of Pathology
Uniformed Services University of the Health Sciences
1982 - 1983

Associate Professor and Coordinator for Medical Genetics
Curriculum
1983 - 1988

Department of Pathology Uniformed Services University of the Health Sciences Professor, Department of Pathology Research Professor, Department of Surgery Coordinator for Medical Genetics Curriculum Director, Tumor Biology Program Uniformed Services University of the Health Sciences	1988 - 1994
Professor of Surgery (Research) Division of Otolaryngology/Head & Neck Surgery Department of Surgery Stanford University Medical Center	1994 - 1996
Professor of Surgery (Consultant) Division of Otolaryngology/Head & Neck Surgery Department of Surgery Stanford University Medical Center	1996-Present
Professor of Otolaryngology Department of Otolaryngology/Head & Neck Surgery Georgetown University Medical Center Lombardi Cancer Center	1996-Present
Professor of Oncology and Otolaryngology Departments of Oncology and Otolaryngology Georgetown University Medical Center Lombardi Cancer Center	1999-Present

HONORS AND OTHER SPECIAL RECOGNITION

Honor Society of Phi Kappa Phi	1972
Special Dissertation Fellow Southern Illinois University	1973 - 1974
Author, two papers in 100 most-cited papers in Life Sciences, Current Contents, November 5, 1984	1982 - 1983
Conference Organizer-International Conference on Molecular Biology of Neoplasia Taipai, Taiwan	1984
Ad Hoc Reviewer for NIH Study Section	1985
One of six awardees, Visiting Scholar Exchange Program, National Academy of Sciences, American Council of Learned Societies and Social Science Research Council	1986 - 1987
Member, Merit Review Committee, USUHS	1987 - 1989
Ad hoc Member, Review Panel for Assessment of Department of Energy research projects on chemical toxicology	1989
Member, Faculty Senate Education Committee, USUHS	1990 - 1991
Member, Editorial Board of Antisense Research and Development	1990 - Present

Member, Steering Committee on Prescribing of Drugs by Military Psychologists	1991
Chairman, Subcommittee for Faculty Resources for the Educational Program, Institutional Self-Study at USUHS	1991 - 1993
Member, Scientific Advisory Committee on Design Study for Life Span Experiments in Mice on Carcinogenesis and Biological Effects of Heavy Charged Particles, NASA	1992 - 1994
Chairman, Subcommittee to Examine Faculty, Middle States Association Reaccreditation Self-Study, USUHS	1992 - 1993
Ad hoc Member, Special Review Committee, Epidemiology, NCI	1992
Author, one Nature paper in top ten most cited papers in medicine Science Watch, September, 1992	1992
Member, Board of Scientific Counselors, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute	1993 - 1995
Member, NASA Life and Microgravity Sciences and Applications Advisory Committee	1994 - Present
Member, Interim ad hoc Board of Scientific Counselors, National Cancer Institute, NIH	1995 - 1996
Chair, Molecular Genetics Study Section, U.S. Army Breast Cancer Research Program	1997
Chair, Experimental Gene Therapy, Program Committee AACR Annual Meeting	1999
Member, Board of Scientific Advisors, National Cancer Institute	1999 - 2004
Member , Editorial Board of Cancer Gene Therapy	1999 - Present
Member, Scientific Program Committee. Chair, Gene Therapy Program NCI-EORTC-AACR Symposium	1999
Distinguished Alumni, Fu Jen University	1999
10th Lecturer, Stewart Lectureship	2000
Member, NASA Focus Group - National Academy of Sciences, Committee on Science, Engineering, and Public Policy	2000
Member, Committee of Scientific Advisors, United States Military Cancer Institute	2001 – Present
Ad hoc member, Experimental Therapeutics I + II, Study Section, NIH	2002
Organizer, Conference on "Tumor Specific Delivery by Non-Viral Systems" Maui, Feb. 2003 Sponsored by NCI	2002-2003
Approximately 10 annual invited lectures at national and international conferences and academic and research institutes	1982 - Present

DISSERTATION TITLE

Comparative Studies of Growth Patterns of Ganjam Virus in CE, BHK and VERO and *Aedes albopictus* Cells

RESEARCH ACTIVITIES

Undergraduate
Insect tissue culture. Studied growth pattern of insect line cells (Bombyx, Aedes and Antheraea) and adapted two lines into hemolymph-free media. Gained some experience in the growth of Japanese Encephalitis Virus in insect cells and newborn mice.
Graduate School
Arboviruses (Togaviruses). Electron microscopy. Compared the growth of VSV in insect cells and chicken embryo fibroblasts. Determined the viral RNA profiles in each cell line.
Characterized Ganjam Virus, an ungrouped arbovirus.
Postgraduate
RNA tumor viruses - interferon effect. Studied interferon's inhibitory effect on the replication of murine leukemia virus. (In Robert M. Friedman's laboratory, National Institutue of Arthritis, Metabolic and Digestive Diseases, NIH).
Molecular genetics. Cloned and characterized murine leukemia and sarcoma viruses. Investigated the origin and the functional organization of Harvey murine sarcoma virus. Molecularly cloned four DNA fragments containing human homologous sequences of v-*ras* (2 Harvey and 2 Kirsten) and demonstrated their oncogenic potentials. Studied potential human oncogenes. (In Douglas R. Lowy's Laboratory, Dermatology Branch, National Cancer Institute, NIH).
Current

1) Molecular genetic basis of familial cancer syndrome and the involvement of human oncogenes and tumor suppressor genes in carcinogenesis.
2) Modulation of oncogene expression by sequence-specific antisense oligonucleotides.
3) Molecular basis of cellular radioresistance and radioprotection.
4) Tumor Suppressor Gene Therapy for Cancer (Head and Neck, Breast and Prostate)
5) Ligand directed, tumor-targeted liposome-based systemic gene delivery

MEMBERSHIP IN ORGANIZATIONS AND PROFESSIONAL AFFILIATIONS

Honor Society of Phi Kappa Phi	1973-
American Association for the Advancement of Science	1983-
Society of Chinese Bioscientists in America	1988-
The Wound Healing Society	1991-
American Association for Cancer Research	1993-
American Society of Gene Therapy	1997-

PUBLICATIONS - ESTHER H. CHANG

1. R. M. Friedman, **E. H. CHANG**, J.M. Ramseur and M.W. Myers. Interferon-directed inhibition of chronic murine leukemia virus production in cell cultures: Lack of effect of intracellular viral markers. **J. Virol. 16**: 569-574 (1975).

2. R. M. Friedman, J.C. Costa, J.M. Ramseur, M.W. Myers, F.T. Jay and **E. H. CHANG**. Persistence of the viral genome in interferon-treated cells infected with oncogenic or nononcogenic viruses. **The J. Infectious Diseases 133**: A43-A50 (1976).

3. R. M. Friedman, F. T. Jay, **E. H. CHANG**, M. W. Myers, J. M. Ramseur, S. J. Mims, T. J. Triche, and P.K.Y. Wong. Interferon-directed inhibition of chronic murine leukemia virus production in cell cultures. In: Control of Neoplasia by Modulation of the Immune System. (M.A. Chirigos, ed.), Raven Press, New York (1977), pp. 347-359.

4. R. M. Friedman, E. F. Grollman, **E. H. CHANG**, L. D. Kohn, G. Lee and F. T. Jay. Interferon and glycoprotein hormones. In: Texas Reports on Biology and Medicine (1977), pp. 326-329.

5. R. M. Friedman and **E. H. CHANG**. Interferon action. Possible mechanisms of antiviral activity. In: Interferons and Their Actions (M. Stewart, ed.) CRC Handbook Series (1977), pp. 145-152.

6. **E. H. CHANG,** S. J. Mims, T. J. Triche, and R. M. Friedman. Interferon inhibits mouse leukemia virus release: An electron microscope study. **J. Gen. Viron. 34**: 363-367 (1977).

7. P. K. Y. Wong, P. H. Yuen, R. Macleod, **E. H. CHANG,** M. W. Myers, and R. M. Friedman. The effect of interferon on *de novo* infection of Moloney murine leukemia virus. **Cell 10**: 245-252 (1977).

8. **E. H. CHANG,** M. W. Myers, P. K. Y. Wong, and R. M. Friedman. The inhibitory effect of interferon on a temperature-sensitive mutant of Moloney murine leukemia virus. **Virology 77**: 625-636 (1977).

9. **E. H. CHANG,** and R. M. Friedman. A large glycoprotein of Moloney leukemia vrius derived from interferon-treated cells. **Biochem. Biophys. Res. Commun. 77**: 392-398 (1977).

10. **E. H. CHANG,** F. T. Jay and R. M. Friedman. Physical and morphological alteration in the membrane of AKR cells following interferon treatment and their correlation with the establishment of the antiviral state. **Proc. Natl. Acad. Sci. 75**: 1859-1863 (1978).

11. **E. H. CHANG,** E. F. Grollman, F.T. Jay, G. Lee, L. D. Kohn and R.M. Friedman. Membrane alterations following interferon treatment. In: Human interferon. W. Alton Jones Cell Science Center, Lake Placid, New York (1978), pp. 85-99.

12. A. K. Bandyopadhyay, **E. H. CHANG,** C. C. Levy and R. M. Friedman. Structura abnormalities in murine leukemia viruses produced by interferon-treated cells. **Biochem. Biophys. Res. Commun. 87**: 983-988 (1979).

13. G. L. Hager, **E. H. CHANG,** H. W. Chan, C. F. Garon, M. A. Israel, M. A. Martin, E. M. Scolnick and D. R. Lowy. Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: Initial structural and biological characterization. **J. Virol. 31**: 795-809 (1979).

14. H. W. Chan, C. F. Garon, **E. H. CHANG**, D. R. Lowy, G. L. Hager, E. M. Scolnick, R. Repaske and M. A. Martin. Molecular cloning of the Harvey sarcoma virus circular DNA intermediates: II. Further structural analyses. **J. Virol. 33**: 845-855. (1980).

15. A. I. Oliff, G. L. Hager, **E. H. CHANG,** E. M. Scolnick, H. W. Chan and D. R. Lowy. Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper independent type C virus. **J. Virol. 33**: 475-486 (1980).

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17. E. **H. CHANG**, J. Maryak, D. M. Wei, T. Y. Shih, R. Shober, H. L. Cheung, R. W. Ellis, G. L. Hager, E. M. Scolnick and D. R. Lowy. Functional organization of the Harvey murine sarcoma virus genome. **J. Virol. 35**: 76-92 (1980).

18. R. W. Ellis, D. DeFeo, J. M. Maryak, H. A. Young, T. Y. Shih, **E. H. CHANG,** D. R. Lowy and E. M. Scolnick. A dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. **J. Virol. 36**: 408-420 (1980).

19. **E. H. CHANG** and D. R. Lowy. Transformation by molecularly cloned Harvey murine sarcoma virus DNA. **J. Supramol. Struc. 9 (Supp. 4)**: 237 (1980).

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27. S. K. Chattapadhyay, **E. H. CHANG**, M. R. Lander, R. W. Ellis, E. M. Scolnick and D. R. Lowy. Selective amplification of *onc* genes in mammalian species. **Nature 296**: 361-363 (1982).

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34. **E. H. CHANG**, M.A. Gonda, M.E. Furth, J.L. Goodwin, S.E. Yu, R.W. Ellis, E.M. Scolnick and D.R. Lowy. Characterization of four members of the p21 gene family isolated from normal human genomic DNA and demonstration of their oncogenic potential. In: Gene Transfer and Cancer, Raven Press, New York (1983), pp. 189-197.

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36. D.R. Lowy, M.A. Gonda, M.A. Furth, R.W. Ellis, E.M. Scolnick and **E. H. CHANG**. The human genes homologous to p21 *ras* viral oncogenes. In: Tumor Viruses and Differentiation, Alan R. Liss, Inc., (1983), pp. 435-444.

37. D. Samid, **E. H. CHANG** and R.M. Friedman. Revertants from interferon-treated mouse cells transformed by a human oncogene. In: The Biology of the Interferon System, Elsevier Science Publishers, (1983), pp. 359-360.

38. M. S. McCoy, J. J. Toole, J. M. Cunningham, **E. H. CHANG**, D. R. Lowy and R. A. Weinberg. Characterization of a human colon/lung carcinoma oncogene. **Nature 302**: 79-81 (1983).

39. S. J. O'Brien, W. G. Nash, J. L. Goodwin, D. R. Lowy and **E. H. CHANG**. Dispersion of the *ras* family of transforming genes to four different chromosomes in man. **Nature 302**: 839-842 (1983).

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41. S. J. O'Brien, W. G. Nash, R. Bauer, **E. H. CHANG** and L. J. Seigel. Trends in chromosomal and oncogene evolution in vertebrates. "Uses and Standardization in Vertebrae Culture Cells" (M. K. Paterson, ed.), *IN VITRO* **Monograph No. 5**: Gaithersburg Tissue Culture Association (1984), pp. 204-214.

42. D. Samid, **E. H. CHANG** and R. M. Friedman. Biochemical correlates of reversion in interferon-treated mouse cells transformed by a human oncogene. **Biochem. Biophys. Res. Commun. 119:** 21-28 (1984).

43. D. Samid, **E. H. CHANG** and R. M. Friedman. Inhibition by interferon of transformation induced by a human *ras* oncogene. **Biochem. Biophys. Res. Commun. 126(1):** 509-516 (1985).

44. D. Samid, Z. Schaff, **E. H. CHANG** and R.M. Friedman. Reduction in *ras* expression accompanies phenotypic reversion of interferon-treated, c-Ha-*ras* oncogene transformed mouse cells. In: The Biology of the Interferon System (H. Kirchner and H. Shellekens, eds.), Elsevier, Amsterdam (1985), pp. 189-198.

45. D. Samid, Z. Schaff, **E. H. CHANG** and R. M. Friedman. Interferon-induced modulation of human *ras* oncogene expression. Endocoids. In: Progress in Clinical and Biological Research, Vol. 192 (H. Lal, F. La Bella and J. Lane, eds.), Alan R. Liss, New York (1985), pp. 265-268.

46. D. Samid, **E. H. CHANG** and R.M. Friedman. Specific inhibition by interferon of oncogene-induced transformation. In: Serono Symposia Publications, Vol. 24 (F. Dianzani and G.B. Rossi, eds.), Raven Press, New York, (1985), pp. 425-422.

47. D. Samid, D.M. Flessate, J.J. Greene, **E. H. CHANG** and R.M. Friedman. Mechanisms of Antioncogenic activity of interferon in the 2-5A System: Molecular and clinical aspects of the interferon-regulated pathway. In: Prigin. Clinical and Biological Research, Vol. 202, (B.R.G. Williams and R.H. Silverman, eds.), Alan R. Liss, New York (1985), pp. 203-210.

48. D. Samid, **E. H. CHANG** and R.M. Friedman. Regulation of *ras*-expression by interferon. In: Proc. Asian Congress Pharmacol., (1985), pp. 343-364.

49. **E. H. CHANG**, J.K. Lin, and P. C. Huang, eds. Molecular Biology of Neoplasia. Academia Sinica, 1985

50. **E. H. CHANG**. Viral and cellular oncogenes. In: Molecular Biology of Neoplasia. (E.H. Chang, J.K. Lin and P.C. Huang, eds.) Academia Sinica - Taipei, Taiwan (1985), pp. 191-203.

51. D. Samid, **E. H. CHANG**, and R. M. Friedman. Biological and morphological characteristics of phenotypic revertants appearing in interferon-treated mouse cells transformed by a human oncogene. **J. Exp. Path. 2(3)**: 211-222 (1985).

52. **E. H. CHANG**, P. L. Morgan, E. Lee-Lawlor, K. Pirollo, E. A. White, P. N. Tsichlis and D. H. Patrick. Pathogenicity of retroviruses containing either normal human c-Ha-*ras* 1 or bladder carcinoma EJ/T24 *ras* gene. **J. Exp. Path 2:** 177-190 (1985).

53. R. L. Stallings, R. Black, B. D. Crawford and **E. H. CHANG**. Assignment of *ras* protooncogenes in Chinese hamster: Implications for linkage conservation. **Cytogenet. Cell Genet. 43:** 2-5 (1986).

54. **E. H. CHANG**, R. Black, T. Masnyk and J.B. Harford. Effect of interferon on growth of A431 cells and expression of EGF receptors. In: Advances in Gene Technology: Molecular Biology of the Endocrine System. (D. Puett, *et al*, eds.), Proc. 18th Annual Miami Winter Symposium, 1986, pp. 370-371.

55. **E. H. CHANG**, R. Black, Z.Q. Zou, T. Masnyk, J. Ridge, P. Noguchi and J.B. Harford. Interferon modulates growth of A431 cells and expression of EGF receptors. In: Interferons as Cell Growth Inhibition and Antitumor Factors. (R.M. Friedman, T. Merigan and T. Sreevalsan, eds.), Alan R. Liss, New York (1986), pp. 335-350.

56. **E. H. CHANG**. Oncogenes and familial cancer syndrome. CAPA 86 Conference Proceedings, College Park, MD, 1986, pp. 21-29.

57. **E. H. CHANG**, K. F. Pirollo, Z. Q. Zou, H. Y. Cheung. E. L. Lawlor, R. Garner. E. White, W. B. Bernstein, J. F. Fraumeni, Jr. and W. A. Blattner. Oncogenes in radioresistant, non-cancerous fibroblasts from a cancer-prone family. **Science 237:** 1036-1039 (1987).

58. **E. H. CHANG**, J. Ridge, R. Black Z. Q. Zou, T. Masnyk, P. Noguchi and J. B. Harford. Interferon-induces altered oncogene expression and terminal differentiation in A431 cells. **Proc. Soc. Exp. Biol. Med. 186:** 319-326 (1987).

59. R. L. Black, Z. P. Yu, D. Brown and **E. H. CHANG**. Modulation of oncogene expression by epidermal growth factor and -interferon in A431 squamous cells. **J. Biol. Regulators Hemeo. Agents 2:** 35-44 (1988).

60. H. Blanche, **E. H. CHANG**, J. Dausset and H. M. Cann. A fragment of the human c-Ki-*ras* 1 pseudogene (HGM9 gene symbol KRASIP), localized to 6p12-p11, detects 3 allele, moderately polymorphic RFLP. **Nucl. Acid. Res. 16:** 1652 (1988).

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63. P. S. Miller, L. Aurelian, K.R. Blake, **E. CHANG**, J.M. Kean, B.L. Lee, S.B. Lin, A. Murakami and P.O.P. Ts'o. Antisense oligonucleoside methyl-phosphonates. In: Current Communications in Molecular Biology. Antisense RNA and DNA (D. Melton, ed.), Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1988, pp. 41-45.

64. **E. H. CHANG**. Specificity of methylphosphonate oligomers as down-modulators for *ras* expression. In: NCI/NIAID Workshop on Anti-Sense Oligonucleotides as Therapeutic Agents, Annapolis, MD, 1987 (1988), pp. 91-96.

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116. K.F. Pirollo, A. Rait, L. Sleer, and **E.H. Chang.** Antisense Therapeutics: From Theory to Clinical Practice. **Pharmacology and Therapeutics (In Press)**

117. Y. J. Jang, K.F. Pirollo, Z. Hao, Y. Chiang, and **E.H. CHANG**. Restoration of the G_1 Block and Apoptotic Pathway in SCCA of the Head and Neck by Adenoviral Vector Mediated p53 Gene Therapy. **Submitted to Carcinogenesis.**

118. L. Xu, K.F. Pirollo, W.H. Tang, L.M. Xiang, A. Rait, D. Ulick, W.A. Alexander and **E.H. CHANG**. Systemic P53 Gene Therapy Using a Tumor-Targeted Adenoviral Vector Results in Radio/Chemo Sensitization and Long-Term Tumor Regression. **Submitted to Science.**

119. A. Rait, K.F. Pirollo , L. Xu, V. Rait, L. Xiang and **E.H. CHANG**, Antisense HER-2 Oligonucleotides Sensitize Human Breast Cancer to Taxotere *In Vitro* and *In Vivo.* **Submitted to Human Gene Therapy.**

120. K B. Bouker, K.F. Pirollo and **E.H. CHANG**, p53: Culprit or Bystander in the Treatment Failure of Radio/Chemotherapy. **Submitted to JNCI.**

121. M.S. Jhaveri, A.S. Rait, J.B. Trepel, **E.H. CHANG**. Antisense oligonucleotides targeted to the human alpha folate receptor sensitize breast cancer cells to doxorubicin treatment *in vitro*. **Submitted to Molecular Cancer Therapeutics.**

THESIS AND DISSERTATION

1. E. H. CHANG. Adaptation of Grace's continuous lines of insect cells to medium containing heterologous serum. Bachelor's Thesis (U.S. Naval Medical Research Unit No. 2, Fu Jen University, Taipei, Taiwan (1968).

2. E. H. CHANG. Comparative studies of growth patterns of Ganjam Virus in CE, BHK and VERO and *Aedes albopictus*cells. Ph.D. Dissertation, Southern Illinois University, Carbondale, Illinois (1974).

PATENT - APPLICATION FILED

1. c-Raf Transgenic Non-Human Mammals.
2. An Automated Method for the Detection of p53 Mutations.
3. Treatment of Tumors by a Combination of Radiation Therapy and Transduction with Polynucleotide Encoding Wild Type p53.
4. Method of Reversal of Resistance to Radiation Therapy and to Chemotherapy in Cancer Cells Using Sequence-Specific Anti-HER-2 Oligonucleotides.
5. Modified Antisense Nucleotides Complementary to a Section of the Human Ha-*ras* Gene.
6. Targeted Liposome Gene Delivery.
7. Compositions and Methods for Reducing Radiation and Drug Resistance in Cells.
8. Systemic Viral/Ligand Gene Delivery System and Gene Therapy.
9. Ligand-PEG "Post-coated" Cationic Liposomes for Targeted Gene Delivery.
10. Antibody Fragment-Targeted Immunoliposomes for Systemic Gene Delivery.
11. A Simplified and Improved Method for Complexing an Antibody Fragment-Targeted Immunoliposome for Systemic Gene Delivery.

RESEARCH GRANTS **Esther H. Chang, Ph.D.**

1. Currently Active Support:

1. National Institutes of Health, A Novel Improvement on Radiotherapy for SCCHN
P.I. 20% Effort on Project
Project Period: 1 APRIL 1999-31 MARCH 2003
Total: $286,320

2. Natl. Foundation for Cancer Research, Chemosensitization of Breast Cancer by Systemic Delivery of Anti-HER2 Oligonucleotides
P.I. 5% Effort on Project
Project Period: 1 OCTOBER 2000- 30 SEPTEMBER 2003
Total: $130,435

3. NIH STTR Phase I Application 1R41 CA91660-01A1. Targeting Stealth™ Liposome for Cancer Gene Therapy.
Jointly with SynerGene Therapeutics, Inc
10% Effort on Project
Requested project period: 1 JUNE 2002 – 31 MAY 2003
Total Requested: $41,143 (Georgetown portion).

4. NIH STTR Phase II Application 2R42 CA80449-2A1 Immunoliposome-Mediated Gene Therapy for Prostate Cancer.
Jointly with SynerGene Therapeutics, Inc.
20% effort on project.
Requested Project Period: 1 SEPTEMBER 2002 – 31 AUGUST 2004
Total Requested: $169,280 (Georgetown University Portion).

5. NCI, decision Network Program, Transferrin-Liposome (Synerlip) Mediated Systemic Gene Delivery for Human Prostate Cancer.
P.I.
Project Period: February 1999-
The Decision Network has chosen our transferrin-lioposome-p53 complex (Synerlipp53) for further development and testing in Phase I clinical trials by the NCI.

6. NCI, Rapid Access to Intervention Development (RAID) Program, Tumor-Specific Targeting of wtp53 by Anti-Transferrin Receptor Single Chain Antibody: A New Therapeutic Strategy for Prostate Cancer Treatment
P.I.
Project Period: 1 APRIL 1999-
The RAID program does not supply funds to the approved projects. The RAID is designed to accomplish tasks that are rate-limiting in bringing discoveries from the laboratory to the clinic. Thus, in support of this project the RAID program is producing, through the use of NCI's development contracts, GLP/GMP grade reagents including the TfRscFv, the liposome and the wtp53 expression plasmid.

2. Past Support:

1. USUHS, Regulation of the Expression of Human c-ras Genes.
1 OCTOBER 1982 - 30 SEPTEMBER 1985.
$60,000 - 3 years. P.I. 10%

2. USUHS, Molecular Cloning of a Tumor Oncogene in a Cancer-Prone Family.
1 OCTOBER 1985 - 30 SEPTEMBER 1989.
$154,125 - 4 years. P.I. 10%

3. NIH, Oncogenes (c-ras) in Human Cancer Induction.
1 MAY 1983 - 30 APRIL 1986.
$160,000 - 3 years. P.I. 40%

4. Medical Applications of Advanced Laser Technology (MAALT). Probing the Molecular Mechanisms of Carcinogenesis.

1 JANUARY 1986 - 31 DECEMBER 1988.
$150,000 - 3 years. P.I. 10%

5. NIH, Oncogenes in Human Cancer Induction.
1 SEPTEMBER 1986 - 31 DECEMBER 1989.
$258,791 - 3 years. P.I. 40%

6. NIH, a program project. Subproject III. Modulation of Tumor Cell Growth. Program project P.I. Paul O. P. T'so, Johns Hopkins University. Program project. Title: Oligonucleotide Analogs as Antiviral/Anticancer Agents.
1 AUGUST 1986 - 31 DECEMBER 1989.
$145,190 - 3 years. Co-P.I. 15%

7. Medical Applications of Advanced Laser Technology (MAALT). Experimental Therapy of Human Colorectal Tumors.
1 JANUARY 1989 - 31 DECEMBER 1990.
$80,000 - 3 years. P.I. 10%

8. NIH, Modulation of Tumor Growth in vitro and in vivo.
1 JULY 1990 - 30 JUNE 1995.
$649,018 - 5 years. P.I. 15%

9. NIH, Oncogenes in Human Cancer Induction.
1 DECEMBER 1989 - 30 NOVEMBER 1994.
$757,798 - 5 years. P.I. 25%

10. USUHS, Inherited Genetic Defects in Li-Fraumeni Syndrome.
1 OCTOBER 1992 - 30 SEPTEMBER 1995.
$81,000 - 3 years. Co-P.I. 5%

11. Naval Medical Research and Development Command. Demonstration of Cytokines and Growth Factors in Wound Healing.
1 APRIL 1991 - 30 SEPTEMBER 1996
$485,400 – 5.5 years P.I. 5%

12. National Foundation for Cancer Research, HU0001, Modulation of the Radiation-Resistant Phenotypes of Tumor Cells by Sequence-Specific Oligonucleotides.
1 OCTOBER 1988 - 30 SEPTEMBER 1999
$638,750-9 years P.I. 10%

13. NIH, CA45158, The Status of Suppressor Genes in a Cancer-Prone Family.
1 DECEMBER 1994 - 30 NOVEMBER 1999
$1,003,887 – 5 years P.I. 30%

14. Genetic Therapy Inc./NOVARTIS, Sensitization of Tumors to Radiation Therapy by Restoration of the G1 Checkpoint.
1 OCTOBER 1997 - 30 SEPTEMBER 1998
$60,000-1 year P.I. 5%

15. NIH STTR Phase I Application 1 RA1 CA80449-01. Immunoliposome-Mediated Gene Therapy for Prostate Cancer.
(Jointly with SynerGene Therapeutics, Inc.)
1 NOVEMBER 1998 - 31 OCTOBER, 1999
$57,600 (Georgetown University Portion) 1 year, P.I. 10%

16. NIH, 5D50 CA58185-06, SPORE in Breast Cancer (Marc E. Lippman, P.I.).
Development Project, p53 Mediated, Tumor-Targeted Sensitization to Chemotherapy and Radiotherapy.
1 SEPTEMBER 1997 - 31 AUGUST 2001
$50,000- 4 year P.I. 10%

17.DOD Concept Award, Systemic Apoptin Gene Therapy for Chemo/Radiosensitization of Breast Cancer
1 SEPTEMBER 2000-31 AUGUST 2001

$50,000- 1 year P.I. 5%

3. Pending Support

1. NIH RO1 Application. Non-Invasive Methods to Assess p53 Gene Therapy Effects.
Submitted on February 1, 2001
15% effort on project
Request Project Period: 1 DECEMBER 2001 – 30 NOVEMBER 2005
Total Requested: $1,513,200

2. NIH RO1 Application. Surrogate End-Points to Assess p53 Therapy in SCCHN.
Submitted on June 1, 2001
10% effort on project (CO-PI)
Requested Project Period: 1 April, 2002 – 31 March, 2006
Total Requested: $1,496,640

3. NIH RO1 Application. Systematic Sensitization of Pancreatic Cancer to Gemzar
Submitted on February 1, 2002
20% effort on project
Requested Project Period: 1 December 2002 – 30 November 2006
Total Requested: $1,864,810

CHURCHILL LIVINGSTONE

Drug Resistance Updates 5 (2002) 11–18

DRUG RESISTANCE Updates

EGF receptor targeting in therapy-resistant human tumors

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Abstract

The development of resistance against cytotoxic or endocrine therapy limits the number of chemotherapeutic compounds used in the clinic. The receptor for EGF (EGFR) is not only involved in survival signaling, cell migration, metastasis formation and angiogenesis, but also confers reduced responses of tumor cells towards cytotoxic compounds or radiation. Clinical trials designed to combine EGFR inhibitors with standard chemo- or radiation therapy have been successful. Elucidation of some of the molecular mechanisms of EGFR-mediated chemoresistance may lead to novel treatment approaches against molecules linked to EGFR signal transduction. In human breast carcinomas, the presence of EGFR correlates with lack of response towards anti-estrogen therapy suggesting the concomitant inhibition of both the receptors for estrogen and EGF to improve breast cancer therapy.

Keywords: EGF receptor; Estrogen receptor; Cell cycle; Apoptosis; Endocrine resistance

1. Introduction

The majority of human carcinomas are either intrinsically chemoresistant or become refractory to chemotherapy upon treatment. The development of chemoresistance is the most significant obstacle towards curative treatment of patients whose primary tumors or distant metastases are not completely removed by surgery. Disrupting chemoresistance and thereby improving current therapeutic protocols is an unmet medical need and has led to intensive studies of the underlying molecular mechanisms of action and resistance.

In recent years it has been elucidated that the EGF receptor (EGFR) not only modulates growth properties of transformed cells, but is causally involved in survival signaling, cell migration, metastasis, and angiogenesis. With the development of EGFR-specific inhibitors like receptor-blocking antibodies or small-molecule inhibitors of the cytoplasmic tyrosine kinase, the tools are available to explore the mechanisms that lead to chemosensitization upon receptor blockade. Successful clinical trials designed to combine EGFR inhibitors with standard chemo- or radiation therapy have been reported. The strategy of targeting EGFR has generated novel clinical anti-cancer agents and has provided tools for analyzing the molecular changes that lead to increased response towards chemo- or radiation therapy. Our increased knowledge of the molecular mechanisms that underlie chemosensitization upon receptor blockade may lead to novel treatment approaches directed against molecules that are linked to EGFR signal transduction. This review focuses on elucidating some of the molecular mechanisms of EGFR-mediated chemoresistance and their clinical consequences. Furthermore, the presence of EGFR correlates in human breast carcinoma with lack of response to anti-estrogen therapy. The rationale for therapies combining blockade of EGFR and of the estrogen receptor are discussed.

1.1. EGFR signal transduction and biology

The EGFR belongs to the type I family of receptor tyrosine kinases. Members of this family include the EGFR as prototype, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Activation of the EGFR upon binding of its ligand(s) induces receptor dimerization followed by autophosphorylation of the cytoplasmic domain at various tyrosine residues. Six ligands are known to activate the EGFR: EGF, TGFα, heregulin, beta-cellulin, heparin-binding EGF-like growth factor and epiregulin. Activation of EGFR can lead to intracellular signal transduction via several pathways (Fig. 1).

Activation of the STAT pathway is initiated by phosphorylation of tyrosine residues 1068 and 1086 of EGFR (Coffer and Kruijer, 1995) and subsequent recruitment and

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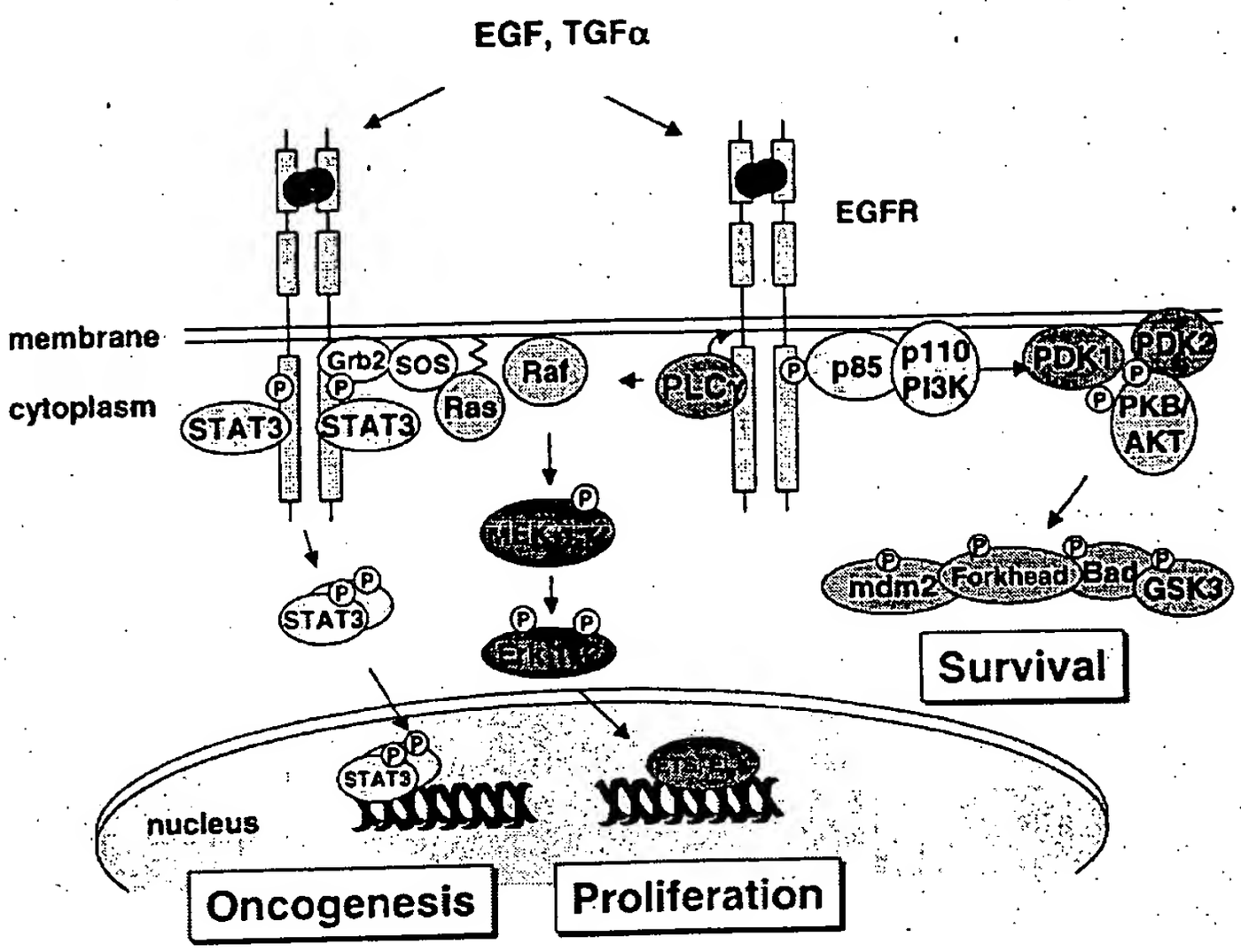

Fig. 1. Oncogenic pathways mediated by EGFR signal transduction. Ligand-induced receptor autophosphorylation results in the recruitment of docking proteins which then divert into strong mitogenic pathways, like the MAP kinase, or survival pathways transduced by the PI 3-kinase. Activation of STAT3 by the EGFR is independent of Janus kinases and is thought to be critical to carcinogenesis (e.g. head and neck cancer), implicating STAT3 as an oncogene. See text for details. Note that PLCγ can phosphorylate T654 in the juxtamembrane region of the EGFR. This leads to a preferential disruption of the MAPK pathway and biases EGFR signaling towards enhanced motility.

phosphorylation of STAT1 (signal transducer and activators of transcription) and STAT3 proteins that are independent from Jaks (Janus kinase) (Bromberg et al., 1999). After phosphorylation, STATs form dimers, translocate into the nucleus and bind to DNA response elements in gene promoter regions, thereby regulating transcription. EGF-dependent STAT signaling is especially prevalent in squamous cell carcinoma of the head and neck (Song and Grandis, 2000).

Activation of the MAP (mitogen activated protein) kinase pathway is initiated following Grb2 binding to phosphotyrosine residues 1068 and 1086 of EGFR via its SH2 (src homology) domain. Grb2 constitutively associates with SOS (son of sevenless) bringing it into close proximity with Ras at the cell membrane. The functional consequence of SOS membrane-recruitment is the activation of Ras through the GDP–GTP exchange activity of SOS. Activated Ras then recognizes and activates the serine/threonine kinase Raf. The signal is further transduced via the kinases MKK1/2 and Erk1/2. Substrates of Erk1/2 comprise other protein kinases like Rsk1,2,3, MAPKAP kinase 2, transcription factors (e.g. c-jun, c-fos), signaling components, cytoskeletal proteins and other targets (Lewis et al., 1998).

EGFR phosphorylation can also activate the class I lipid kinase phosphoinositide 3-kinase (PI3K) which generates phosphatidylinositol 3-phosphates that serve as membrane anchors for PH domain-containing proteins like PKB (protein kinase B)/Akt, PDK1 (phosphoinositide-dependent kinase 1), or Btk (Bruton's tyrosine kinase) like kinases (Leevers et al., 1999). One of the most prominent proteins activated by the PI3K pathway is protein kinase B/Akt. Many proteins known to be involved in modulation of apoptosis are phosphorylated by Akt, among them caspase-9, Bad, forkhead transcription factors, mdm2 (mouse double minute 2) or GSK3 (gylcogen synthetase kinase 3). Phosphorylation of Akt substrates generally modulates their properties towards an anti-apoptotic function. Constitutive activation of Akt has been described in a wide variety of human cancers (Bognard et al., 2001).

In addition to STATS, the MAPK pathway and PI3K, phospholipase Cγ (PLCγ) is able to transduce EGFR activity. After binding to pY992, PLCγ activates second messenger systems (e.g. diacylglycerol) that are required for enhanced cell motility.

In human tumors but not in adjacent normal tissues an EGFR mutant (EGFRvIII) has been found, that lacks exons 2–7 in the extracellular domain, thus deleting the ligand binding domain and exhibiting constitutive kinase activity (Moscatello et al., 1998). In cells expressing this mutant, constitutive activation of PI 3-kinase is observed.

2. EGFR inhibition and the EGFR family

The function of EGFR is closely linked to its family members by sharing either ligands or by cross activation

via ligand-driven receptor heterodimerization. Studies using small molecular EGFR tyrosine kinase inhibitors like AG-1478 and AG-1517, ZD1839 (Iressa), CP 358774 or PD 153035 have revealed that specific inhibition of EGFR activity can concomitantly lead to signal transduction inhibition of other HER-family members (Arteaga et al., 1997; Moasser et al., 2001; Rusnak et al., 2001). Close investigation of the quinazoline-derived inhibitors AG-1478 and AG-1517 revealed that the compounds induced increased formation of EGFR dimers with inactive kinase in the absence of ligand (Arteaga et al., 1997). The formation of kinase-inactive EGFR-homodimers in the absence of ligand could also be described using other quinazoline derivatives, e.g. Iressa and PD 153035 (Lichtner et al., 2001). AG-1478 and AG-1517 could even provoke the formation of kinase-inactive heterodimers of EGFR and ErbB2 (Arteaga et al., 1997). Although Iressa selectively inhibits EGFR activity, tumors with ErbB2 overexpression are particularly sensitive to it (Moasser et al., 2001). Thus, treatment of ErbB2-overexpressing tumors with Iressa resulted in reduced basal phosphorylation of EGFR, ErbB2 and ErbB3, maybe due to inhibition of phosphorylation of receptor heterodimers, which are thought to be driven by EGFR phosphorylation. Most interestingly, inhibition of EGFR by Iressa caused profound downregulation of the PI3K/Akt signaling pathway attributable to dephosphorylation of ErbB3. Other tyrosine kinase inhibitors with dual inhibitory activities on EGFR and ErbB2 are created and exhibit profound tumor inhibitory activities in human xenografts expressing both receptors (Rusnak et al., 2001).

3. Modulation of chemosensitivity by EGFR signaling

Approximately 60% of all tumors are considered intrinsically chemoresistant, whereas the majority of the remaining acquire resistance during therapy. If residual tumor mass remains after surgery, then drug resistance becomes the most significant obstacle hindering the successful treatment of cancer patients. Although many drug resistance mechanisms have been discovered in experimental systems, only few of these mechanisms have been observed in the clinic.

The role of the EGFR in the modulation of tumor chemosensitivity has been debated extensively in the past. In experimental systems using breast cancer cells, downregutaion of the EGFR resulted in decreased sensitivity towards cisplatin but not to other chemotherapeutics (Dixit et al., 1997). The majority of reports show a significant growth inhibition and/or chemosensitization upon EGFR blockade. Tools for EGFR inhibition have been receptor blocking antibodies like C225, Mab E7.6 (Yang et al., 1999). or inhibitors of EGFR tyrosine kinase of which Iressa is currently the most advanced small molecule compound in the clinic.

The first support for the concept of chemosensitization by EGFR blockade was provided by studies in which EGFR-blocking antibodies synergized in vivo with cisplatin or doxorubicin in human tumor xenografts (Aboud-Pirak et al., 1988; Baselga et al., 1993; Fan et al., 1993). Similarly, the combination of antibody plus radiation produced synergistic anti-tumor effects in vitro and in vivo (Huang and Harari, 2000). Interestingly, Mab E7.6.3 was able to eradicate tumor xenografts without the need of concomitant chemotherapy. The growth inhibitory effects of EGFR blockade could be further enhanced by paclitaxel in mice bearing metastatic human bladder carcinoma (Inoue et al., 2000).

The success of this combination treatment was also shown with Iressa (Ciardiello et al., 2000). Strikingly, the degree of potentiation of cytotoxic action was not dependent on the degree of EGFR overexpression (Sirotnak et al., 2000). The basis for this result is not known, however, it has been demonstrated that Iressa is able to promote the formation of inactive EGFR/EGF signaling complexes independent of EGFR numbers (Lichtner et al., 2001). Synergistic effects between Iressa and multiple classes of chemotherapeutic agents were observed, with taxanes being most effective. In this aspect, it is interesting to note that in experimental systems EGFRvIII-expressing tumor cells exhibit altered β-tubulin isotype expression rendering cells less sensitive to paclitaxel (Montgomery et al., 2000). This concept of chemo- and radiosensitization has been successfully transferred to the clinic (see below).

A variety of cellular pathways were analyzed in order to elucidate the mechanisms by which EGFR inhibition sensitizes tumor cells to chemo- or radiation therapy. Inhibition of the EGFR by blocking antibodies results in the upregulation of the cdk inhibitor protein $p27^{Kip1}$, which is known to mediate cell cycle arrest and growth inhibition (Fan et al., 1997). Paradoxically, overstimulation of the EGFR can result in the upregulation of another cell cycle regulator, $p21^{Waf1}$ (Fig. 2). These observations, that modulation of EGFR signaling by ligands or blocking antibodies can induce the expression of cdk inhibitors highlighted these molecules as potential modulators of chemosensitivity. Upregulated expression of $p27^{Kip1}$ has been in general correlated with a lower tumor grade and with a good prognosis for the patient (Newcomb et al., 1999; Mizumatsu et al., 1999), which argues in favor of $p27^{Kip1}$ being a major effector of therapeutic efficacy of EGFR blockade therapy. A wealth of information exists about $p21^{Waf1}$ and $p27^{Kip1}$ and their modulation of tumor cell sensitivity. However, while EGFR blockade in most systems is described to correlate with chemosensitization (see below), both cdk inhibitors have been shown to induce chemoresistance towards a wide variety of chemotherapeutic agents when ectopically overexpressed in an inducible fashion (Schmidt and Fan, 2001; Schmidt et al., 2001). It can be concluded that the upregulation of $p27^{Kip1}$ expression upon EGFR blockade may account for growth inhibition in vitro and in vivo, but that it probably does not confer chemosensitization towards chemo- or radiation therapy.

The concept that a growth factor under certain circumstances (like chemotherapeutic treatment) can turn into a

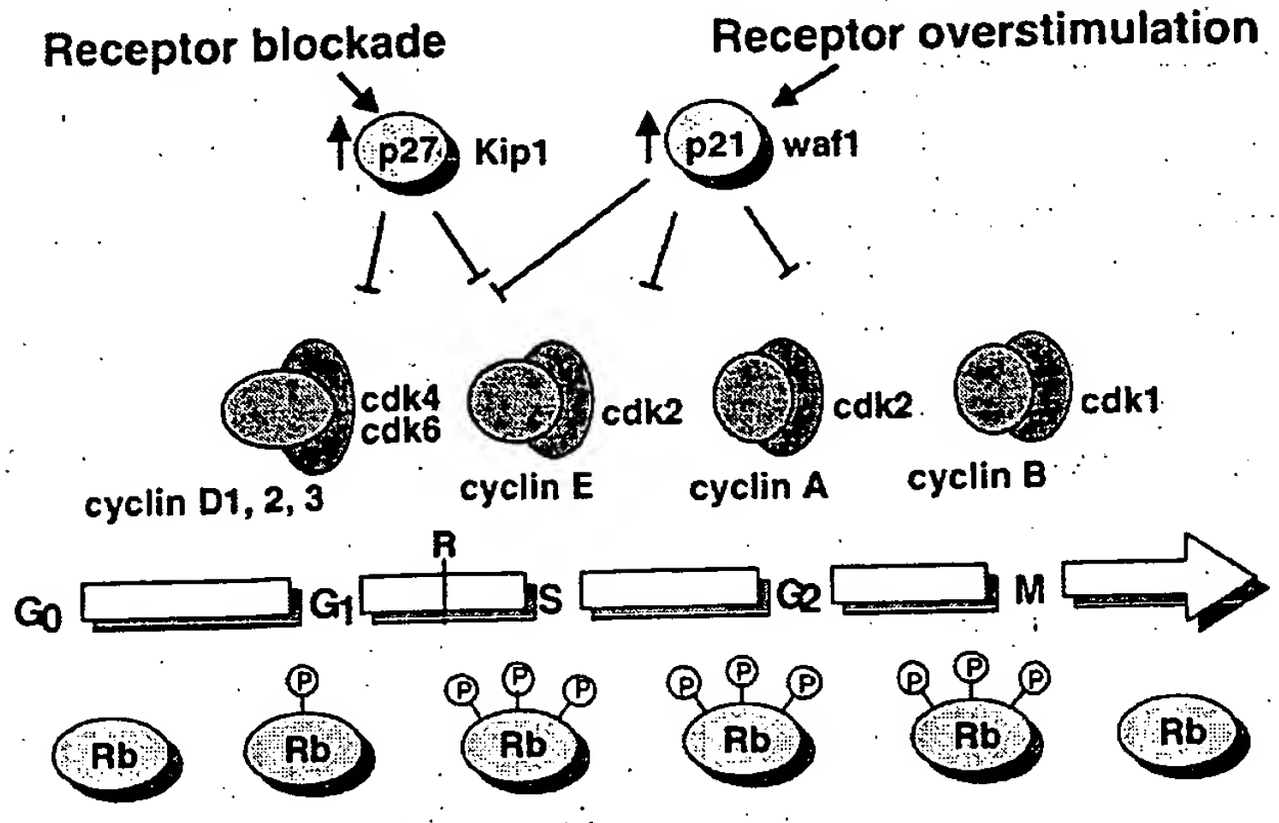

Fig. 2. Paradoxical upregulation of cdk inhibitors by either EGFR blockade or overstimulation. In certain cellular systems EGFR blockade with inibitory antibodies can lead to upregulation of $p27^{Kip1}$, while receptor overstimulation can induce expression of $p21^{Waf1}$. Expression of these cdk inhibitors results in the hypophosphorylation of the retinoblastoma protein and thus in inhibition of cell cycle progression. Note that upregulation of $p27^{Kip1}$ probably accounts for the growth inhibitory properties of anti-EGFR antibodies, but that it is unlikely to confer chemosensitivity.

survival factor is under intensive study. Actually, apoptosis after EGFR blockade alone is observed very rarely. One cellular model studied is the Difi colon adenocarcinoma cell line in which C225 induces caspase-8 activation, followed by caspase-9 and caspase-3 activation finally resulting in induction of apoptosis (Liu and Fan, 2001).

The concept of EGFR signaling as survival pathway has been very elegantly shown in transgenic mice expressing a dominant form of SOS (Sibilia et al., 2000). These transgenic animals develop skin papillomas with 100% penetrance. Tumor formation was inhibited in mice with an EGFR null background, and EGFR null fibroblasts were not transformed upon transfection with plasmids encoding RasV12 or dominant SOS. EGFR null cells revealed increased apoptosis and a significantly decreased Akt phosphorylation. As described above, the PI3K pathway connects a multitude of survival signals from receptor tyrosine kinases to PKB/Akt. Akt itself is a key modulator of anti-apoptotic signaling. It phosphorylates central components of the apoptotic pathway thereby shifting the apoptotic threshold towards survival. Mechanisms include inactivation of Bad and caspase-9, activation of IKK, thereby inducing NFkB transcription of anti-apoptotic genes, induction of cytoplasmic localization of $p21^{Waf1}$ and $p27^{Kip1}$, promotion of nuclear entry of mdm2, thereby inhibiting p53, or activation of telomerase (Testa and Bellacosa, 2001). Several other studies link the EGFR-mediated activation of Akt via PI3K with suppression of apoptosis, both in tumor (Gibson et al., 1999; Wang et al., 2000) and in normal cells (Wan et al., 2001).

Furthermore, it is well established that EGFR stimulation can induce the release of several growth factors, among them VEGF and bFGF, both known for their angiogenic properties. Thus, it is not surprising that EGFR inhibition by antibodies or Iressa resulted in inhibition of the synthesis of these angiogenic factors (Perrotte et al., 1999; Ciardiello et al., 2001). EGFR blockade plus radiation therapy led to decreased expression of markers for angiogenesis like VEGF and factor VIII (Huang and Harari, 2000). Supporting this hypothesis is the C225-induced apoptosis of vascular endothelial cells in a human pancreatic tumor xenograft model (Bruns et al., 2000). Thus, inhibition of a growth factor cascade initiated by EGFR stimulation and leading to VEGF production may be one of the contributing factors for the in vivo efficacy of anti-EGFR monotherapy. Closely linked to angiogenesis is the inhibition of invasion and metastases by EGFR blockade. For example, EGFR blockade was associated with decreased metalloproteinase-9 expression in an orthotopic bladder carcinoma model (Inoue et al., 2000; Perrotte et al., 1999).

3.1. Therapeutic implications

The concept of combining chemotherapy or radiation therapy with modulators of EGFR signaling in order to sensitize tumors towards cytotoxic impacts has been successfully introduced into the clinic. In a phase Ib clinical trial, C225 was combined with cisplatin for the treatment of patients with recurrent squamous carcinoma of the head and neck. The overall response rate was about two-thirds of the patients including some complete responses (Shin et al., 2001). Another phase I study also with advanced head and neck cancer patients reported the combined treatment with the antibody plus radiation therapy. All 16 patients enrolled displayed objective response; among them were 13 patients with complete remissions (Robert et al., 2001). Randomized phase III trials are now underway to determine the role of C225 combined with radiation versus radiation therapy alone.

The most clinically advanced EGFR tyrosine kinase inhibitor is the orally available quinazoline derivative Iressa (Swaisland et al., 2001). A number of phase II studies in patients with solid tumors and a phase III study in patients with NSCLC are currently in progress. Most of those studies are designed to give Iressa alone as a therapeutic intervention, while a phase III study combines Iressa with cisplatin, etoposide and paclitaxel. Two more 4-anilinoquinazoline analogues (one with reversible and one irreversible binding characteristics) have been evaluated clinically as anti-cancer drugs (Denny, 2001).

4. Endocrine-resistant breast tumors

In the control of normal mammary gland growth and development as well as breast cancer promotion both steroid hormones and peptide growth factors play an important role. Clinically, the presence of EGFR has been reported to be indicative of poor prognosis and to correlate with lack of response to endocrine therapy in recurrent breast cancer. Breast tumors are reported to express estrogen receptors (ER)α, ERβ and progesterone receptors (PR)-A and PR-B. While ERα and ERβ are both stimulated by estrogens, the PR-A and -B are estrogen-induced and thus indicate functional ERs. In primary breast carcinoma, ERα seems to be more instrumental than ERβ, but shift in expression to ERβ in endocrine-resistant breast tumors has been reported (Speirs et al., 1999). Since no data are available yet on the concomittant expression of EGFR and ERβ this article will focus on the interdependence of EGFR and ERα expression.

Upon binding of the ligand estradiol, ERs bind to DNA response elements in gene promoter regions thus regulating transcription. However, both ERs also activate gene transcription by binding via protein–protein interactions with AP-1 sites in the promoter of target genes and exert differential effects (Paech et al., 1997).

The non-steroidal anti-estrogen tamoxifen competes with estradiol for binding to ER and has been used by more than 10 million patients (Clarke et al., 2001). Response rates of up to 70% are reported in patients expressing ER and PR; however, most responsive tumors will eventually acquire resistance. The new pure steroidal anti-estrogen Faslodex is currently in phase III studies and shows effectiveness in tamoxifen-resistant tumors.

4.1. De novo anti-estrogen resistance

The most important factor in de novo resistance is lack of expression of steroid hormone receptors and/or simultaneous expression of EGFR or ErbB2 in breast tumors. While ErbB2 is overexpressed in approximately 20% of breast cancers due to gene amplification, this has been found in less than 3% of tumors for EGFR (Sharma et al., 1996). In a study by Bolla et al. (1992) that included 303 patients, 42% were ER-positive, 32% expressed both receptors, 18% expressed only EGFR and 8% were negative for both. Clinically, the presence of EGFR has been reported to be indicative of poor prognosis and to correlate with lack of response to endocrine therapy in recurrent breast cancer. Thus, clinical response of human breast tumors to first-line tamoxifen therapy correlated with ER status, but there was no benefit of hormonal therapy when correlated with either Ki-67 or EGFR positivity (Archer et al., 1995). Furthermore, when response to tamoxifen treatment was correlated with levels of EGFR expression in 106 patients, tumors with high EGFR expression had an extremely low response rate concerning time to disease progression and survival when compared with tumors with low or no expression of EGFR (Nicholson et al., 1994). When human breast tumors were molecularly investigated for expression of EGFR and ER using several analytical methods (levels of RNA, protein, ligand binding) then an inverse correlation could be found in most of 40 separate studies involving 5232 patients (Klijn et al., 1992). Furthermore, it was shown that expression of ER and EGFR are mutually exclusive in individual tumor cells when tested by simultaneous dual immunocytochemistry of breast carcinomas (Sharma et al., 1994; van Agthoven et al., 1994). Interestingly, adjacent normal breast epithelial cells expressed both receptors in the same cell (van Agthoven et al., 1994; Sharma et al., 1997). In the rodent mammary glands, TGFα can mimic some of the effects induced by estradiol in the formation of the terminal-end bud structures (Hilakivi-Clarke et al., 1997). Similarly, coexpression of EGFR and ER in normal cells of the mouse uterus had been reported in elegant studies by Ignar-Trowbridge et al. (1992).

While the inverse relationship between ER and EGFR in human breast tumors is a consistent observation, the precise mechanisms responsible for this correlation are not fully understood. Several studies suggest that the inverse relationship between ER and EGFR is determined by regulation of one receptor gene by the product of the other and/or reciprocal control by a common regulator which has opposite effects on the two receptors at the transcriptional or post-transcriptional level (Sharma et al., 1996). Thus, when T47D and MCF7 breast cancer cell lines were either treated with progestins, 12-*O*-tetra-decanoylphorbol-13-acetate or sodium butyrate then reciprocal transcriptional modulation of ER and EGFR was found. When ER levels were diminished by using antisense constructs then increased expression of EGFR was observed in human breast cancer cell lines (deFazio et al., 1997).

When activation status of the ER was affected, a number of studies showed that estradiol deprivation or the use of an anti-estrogen in ER-positive breast cancer cell lines resulted in increased expression of EGFR. Chrysogelos et al. (1994) demonstrated that regulation of the EGFR gene in ER-positive breast cancer cell lines involves a number of interactions between positive regulatory factors and repressors. In a recent study by McLelland et al. (2001), long-term treatment of MCF7 cells in the presence of the pure anti-estrogen Faslodex led to the emergence

of a subline capable of growing in the presence of the compound. The cells still responded to estradiol, expressed diminished mRNA and protein levels of ERα and showed reduced ER–ERE-mediated cell signaling pathways. Closer investigation of the cells indicated that they had upregulated EGFR and MAPK, components of a pathway signaling through AP-1 sites. Most interestingly, growth of anti-estrogen-resistant, EGFR-overexpressing cells could be inhibited by use of the specific EGFR tyrosine kinase inhibitor Iressa. Even more importantly, the development of anti-estrogen-resistant MCF7 cells was prevented by concomittant exposure of cells to Faslodex plus Iressa.

Conversely, when the EGFR signaling pathway in ER-positive breast cancer cells was enhanced those studies strongly suggested a link between increased growth factor signaling and the acquisition of anti-estrogen resistance. Thus, the stable transfection of either MCF7 or ZR-75-1 breast cancer cells with the EGFR gene and subsequent increased expression of EGFR protein has been shown to result in loss of hormone responsiveness of the cells (van Agthoven et al., 1992; Miller et al., 1994).

4.2. Acquired anti-estrogen resistance

No loss of ER can be found in tumors of tamoxifen-resistant patients (Robertson, 1996) and second-line endocrine responses occur in many acquired-resistant breast cancer patients. So far, the occurence of ER mutants or ER splice variants could not be shown convincingly; a shift to expression of ERβ has been suggested and awaits further confirmation in larger tumor samples (Speirs et al., 1999). Examination of breast tumors from patients relapsing while on tamoxifen could not detect significant changes in the expression of EGFR, irrespective of the initial response. However, the inverse relationship between EGFR and ER was maintained at relapse on tamoxifen (Newby et al., 1997). Possibly, upregulation of EGFR in those tumors might have been missed since a mutually repressive phenotype between ER and EGFR most probably is not a stable one, and thus time between withdrawal of tamoxifen and analysis of tumor samples is very critical. Despite the failure to clearly see upregulation of EGFR or other growth factor receptors (e.g. ErbB2) in tamoxifen-resistant breast tumors, most investigators postulate that alterations in growth factor signalling may have occured (Clarke et al., 2001; Nicholson and Gee, 2000). These changes may either still facilitate functions of ER in a lowered endocrine environment, activate ER ligand-independently or enable the cell to circumvent ER-dependent signalling.

4.3. Therapeutic implications

The inverse correlation in the expression of ER and EGFR in human breast tumors is consistently reported while the basis for this is still unclear. If expression of ER and EGFR are stable phenotypes in human breast tumors, then the response of those tumors to an anti-estrogen is predicted in Fig. 3, and would be improved by cotreatment with an EGFR inhibitor. If expression of ER and EGFR are mutually repressive as suggested in experimental systems, then the combination of

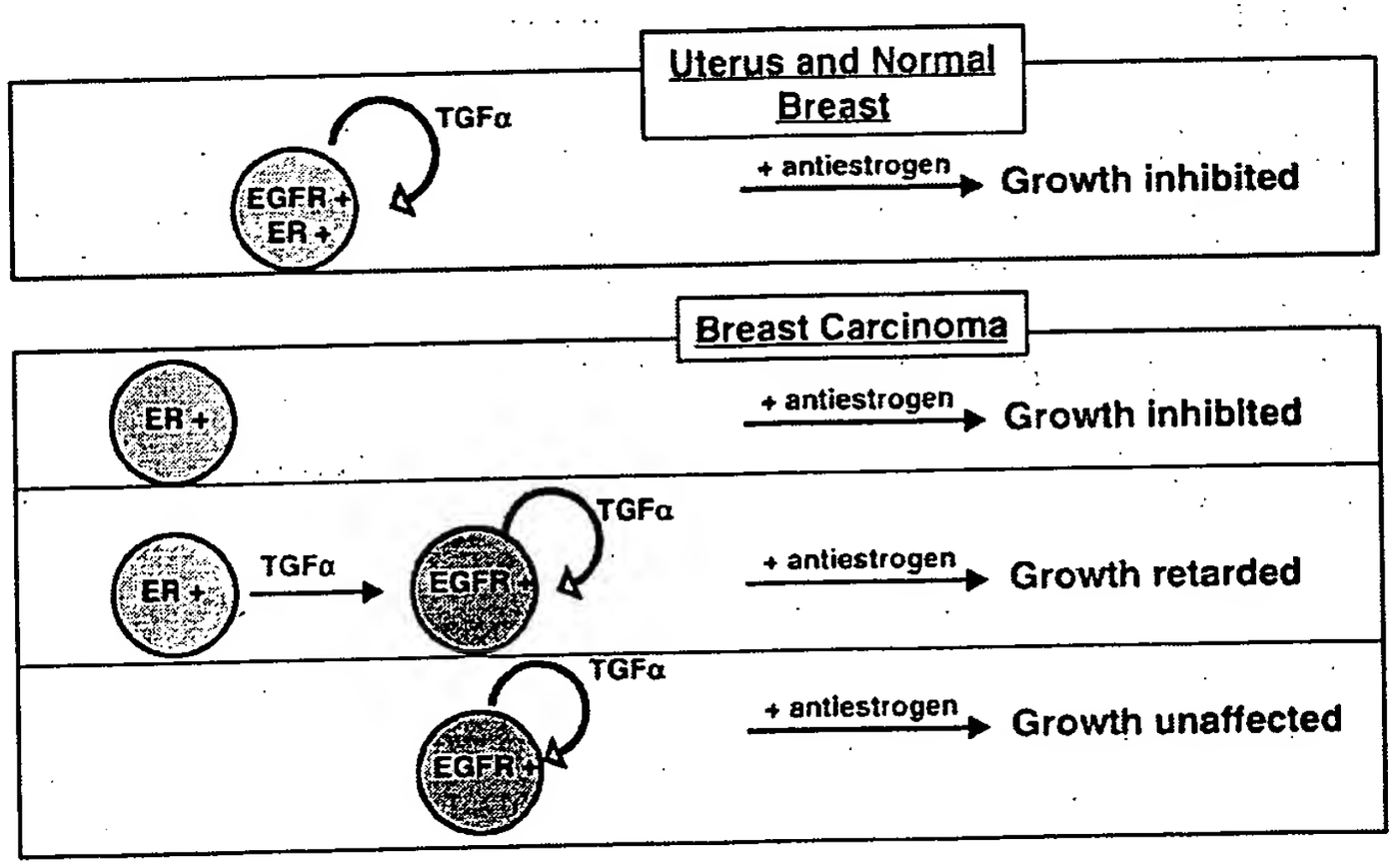

Fig. 3. Tumor cell populations in human breast carcinoma and response to anti-estrogen treatment. In the normal breast and uterus epithelial, cells coexpressing EGFR and ER exist and treatment with an anti-estrogen results in growth inhibition. In human breast tumors containing only ER-positive cells endocrine treatment will result in tumor growth inhibition. In breast cacinomas containing subpopulations of tumor cells expressing ER or EGFR endocrine treatment will result in growth inhibition of ER-positive tumor cells and growth retardation of EGFR-positive cells if dependent on paracrine supply of TGFα from ER-positive cells. EGFR-positive tumor cells with sufficient production of autocrine TGFα are unaffected by an anti-estrogen. In summary, growth of a tumor with mixed subpopulations will be retarded by endocrine treatment, while growth of a tumor consisting only of EGFR-positive cells will be unaffected by endocrine treatment.

an anti-estrogen with an EGFR inhibitor would be even more efficacious. This concept awaits proof in the clinic.

5. Conclusions

A wealth of information exists on the plethora of signaling pathways that are relayed by the EGFR. These pathways can help tumor cells acquire properties allowing them to thrive under conditions that would normally induce the onset of apoptosis in non-transformed cells. Probably, it is the diversity of EGFR signaling that has made this pathway so fundamentally important in clinical oncology. The success of targeted therapies aimed at EGFR blockade, therefore, is most likely founded in the fact that inhibition of a central molecule results in the suppression not of a linear pathway, but several branched cascades important for tumor growth and survival. Still, for the translation of our increased knowledge on EGFR signal transduction it is obvious that inhibition of the receptor alone in most cases does not suffice to induce a profound therapeutic response in cancer patients.

From all the preclinical and clinical data dealing with EGFR inhibition and anti-tumoral efficacy, it has become clear that these new EGFR-targeted drugs exert their full therapeutic potential not as single agents, but in combination with standard chemotherapy (Ryan and Chabner, 2000; Shah and Schwartz, 2000).

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Abstract
The development of resistance against cytotoxic or endocrine therapy limits the number of chemotherapeutic compounds used in the clinic. The receptor for EGF (EGFR) is not only involved in survival **signal**ing, cell migration, metastasis formation and angiogenesis, but also confers reduced responses of tumor cells towards cytotoxic compounds or radiation. Clinical trials designed to combine EGFR inhibitors with standard chemo- or radiation therapy have been successful. Elucidation of some of the molecular mechanisms of EGFR-mediated **chemoresistance** may lead to novel treatment approaches against molecules linked to EGFR **signal transduction**. In human breast carcinomas, the presence of EGFR correlates with lack of response towards anti-estrogen therapy suggesting the concomitant inhibition of both the receptors for estrogen and EGF to improve breast cancer therapy. [References: 62]

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Chapter 5

THE ROLE OF SIGNAL TRANSDUCTION PATHWAYS IN DRUG AND RADIATION RESISTANCE

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1. THE MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

"MAPK" was first reported by Sturgill and Ray in 1986[1]. This protein kinase was originally described as a 42-kDa insulin-stimulated protein kinase activity whose tyrosine phosphorylation increased after insulin exposure, and which phosphorylated the cytoskeletal protein MAP-2 (hence "MAP" kinase). Contemporaneous studies by Boulton and Cobb identified an additional 44-kDa isoform of MAPK, which they named ERK1 (extracellular signal regulated kinase)[2]. Since many growth factors and mitogens could activate MAPK, the acronym for this enzyme has subsequently been considered to denote mitogen-activated protein (MAP) kinase. In the following years, additional studies demonstrated that the p42/p44 MAPKs regulated another protein kinase activity ($p90^{rsk}$)[3], and that they were themselves regulated by a protein kinase activity originally designated MKK (MAPK kinase)[4,5].

MKK phosphorylates the MAPKs on tyrosine and threonine residues and became the first biochemically characterized dual specificity (threonine/tyrosine) protein kinase[4–6]. This enzyme is also often referred to as MEK (mitogen activated/extracellular regulated kinase). Shortly after the discovery of MKK1, a second isoform of this enzyme was identified (MKK1 and MKK2)[7]. MKK1/2 were also found to be regulated by reversible phosphorylation, and within 6 months of the discovery of MKK2, the protein kinase responsible for catalyzing MKK1/2 activation was discovered, the

proto-oncogene Raf-1[8,9]. More recently, it has been suggested that other enzymes at the level of MKK1/2 can phosphorylate and activate p42/44 MAPK; e.g., RIP2[10]. RIP2 plays a role in TNFα-induced, but not EGF-induced, MAPK activation and may play a protective NFκ-B-activating role[10].

Raf-1 is a member of a family of serine-threonine protein kinases termed Raf-1, B-Raf, and A-Raf[11,12]. Each protein consists of an NH_2-terminal domain (termed CR1), a COOH-terminal catalytic domain (termed CR3), and a central domain that is heavily phosphorylated *in vivo* (termed CR2). All "Raf" family members can phosphorylate and activate MKK1/2, although the relative ability of each member to catalyze this reaction varies (B-Raf > Raf-1 > A-Raf)[13,14]. Raf kinases thus act at the level of a MAPK kinase kinase (MAPKKK). Several studies demonstrated that the CR1 domain of Raf-1 could reversibly interact with the Ras proto-oncogene in the plasma membrane and that the ability of Raf-1 to associate with Ras was dependent upon the Ras molecule being in the GTP-bound state[15,16]. Other findings proved that the ability of Raf-1 to be activated depended upon Raf-1 translocation to the plasma membrane[17–20]. The regulation of Raf-1 activity appears to be very complex, with several mechanisms coordinately regulating activity when in the plasma membrane environment. Stokoe and McCormick have demonstrated that association of Raf-1 with Ras is sufficient for partial stimulation of Raf-1 activity[21]. More recently, the binding of 14-3-3 proteins to phospho-serine residues (S259, S621) in Raf-1 have been suggested to play a role in Raf-1 activation[22–24]. Phosphorylation of S338 by PAK enzymes has more recently been shown to play a role in the activation process[25]. Other investigators have suggested that another lipid second messenger, ceramide, may also be able to play a role in Raf-1 activation[26,27], although this is disputed[28,29]. Data from several laboratories have suggested that protein serine/threonine and tyrosine phosphorylations play a role increasing Raf-1 activity when in the plasma membrane environment[29–31]. Other studies have also suggested that PKC (protein kinase C) isoforms can directly regulate Raf-1 activity[32,33]. Phorbol esters and the macrocyclic lactone bryostatin 1 can activate PKC, and have been shown to activate Raf-1 and the MAPK cascade in many cell types[34,35]. At the same time that Raf-1 was shown to associate with Ras, it was found that growth factors, via their plasma membrane receptors, stimulate GTP for GDP exchange in Ras using guanine nucleotide exchange factors[36,37]. Thus, over an interval of ~9 years, a "MAPK" pathway was delineated from plasma membrane growth factor receptors, through guanine nucleotide exchange factors and the Ras proto-oncogene, to the Raf-1/MKK/MAPK/$p90^{rsk}$ (Figure 1). During this period, other studies had begun to link growth factor induced MAPK and $p90^{rsk}$ activation to the ability of these mitogens to regulate transcription factor activities within the nucleus[38,39]. The relative ability of MAPK signaling to mediate increased activity of transcription factors is under intensive study because it appears that many signaling pathways, e.g., the JNK pathway, coordinately regulate transcription factor activities and gene expression along with the classical MAPK pathway[40–43].

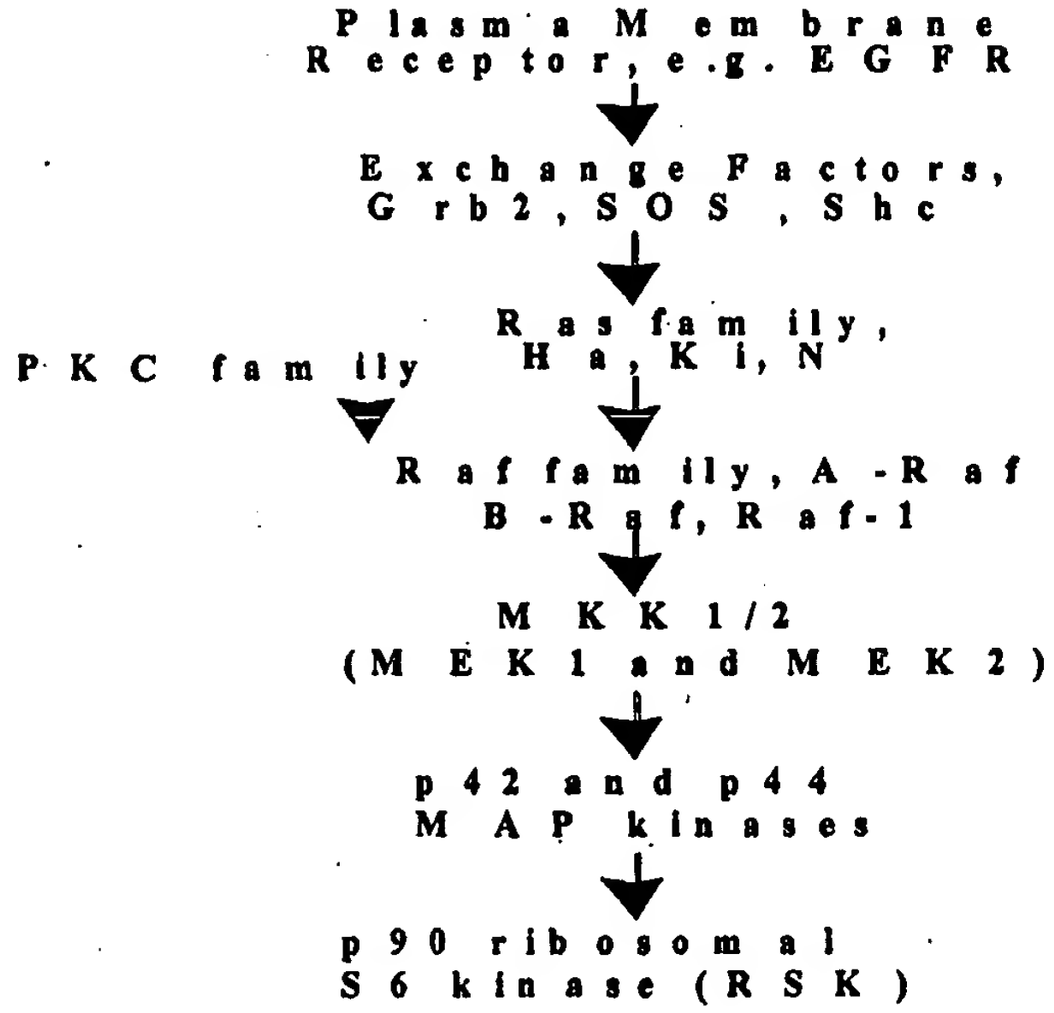

Figure 1. The MAPK pathway in mammalian cells.

2. THE C-JUN NH_2-TERMINAL KINASE (JNK)/STRESS ACTIVATED PROTEIN KINASE (SAPK) PATHWAY

The c-Jun NH_2 terminal kinase (JNK) pathway was discovered and described in the early to mid 1990's[44,45]. JNK1/2 were initially described biochemically to be a stress-induced protein kinase activity that phosphorylated the NH_2-terminus of the transcription factor c-Jun; hence the pathway is also often called the stress activated protein kinase (SAPK) pathway. Multiple stresses increase JNK1/2 activity, including UV- and γ-irradiation, cytotoxic drugs and reactive oxygen species (H_2O_2). Phosphorylation of the NH_2-terminal sites Ser63 and Ser73 in c-Jun increases its ability to transactivate AP-1 enhancer elements in the promoters of many genes[46,47]. It has been recently suggested that JNK can also phosphorylate the NH_2-terminus of c-Myc, enhancing its activity, potentially playing a role in both proliferative and apoptotic signaling[48]. In a similar manner to the previously described MAPK pathway, JNK1/2 activities were regulated by dual threonine and tyrosine phosphorylations which were found to be catalyzed by a protein kinase analogous to MKK1/2, termed stress-activated extracellular regulated kinase 1 (SEK1), also called MKK4[49]. An additional isoform of MKK4, termed MKK7, was

subsequently discovered[50]. As in the case of MKK1/2, MKK4/7 were also regulated by dual serine phosphorylation. In contrast to the MAPK pathway, which appears to primarily utilize the three protein kinases of the Raf family to activate MKK1/2, at least ten protein kinases are known to phosphorylate and activate MKK4/7, including MKKK1-4, TAK-1 and Tpl-2[51]. The agonist and cell type specificity of each JNK pathway MAPKKK enzyme in the activation of this pathway is currently under intense investigation.

Upstream of the MAPKKK enzymes are another layer of JNK pathway protein kinases, e.g., Ste20-homologues and low molecular weight GTP-binding proteins of the Rho family, in particular Cdc42 and Rac1 (Figure 2)[52]. It is not clear how growth factor receptors, e.g., EGF receptor, activate the Rho family low molecular weight GTP-binding proteins; one mechanism may be via the Ras proto-oncogene, whereas others have suggested via PI_3 kinase and/or protein kinase C isoforms (Figure 2)[53,54]. In addition, other groups have shown that agonists acting through the tumor necrosis factor alpha (TNFα) receptor, *via* sphingomyelinase enzymes generating the lipid second messenger ceramide, can activate the JNK pathway by mechanism(s) which may also act through Rho family GTPases[55]. Definitive answers to all of these questions await further investigation. In the following sections, potential roles in the control of growth, proliferation, cell survival and DNA repair for the JNK and MAPK pathways are examined.

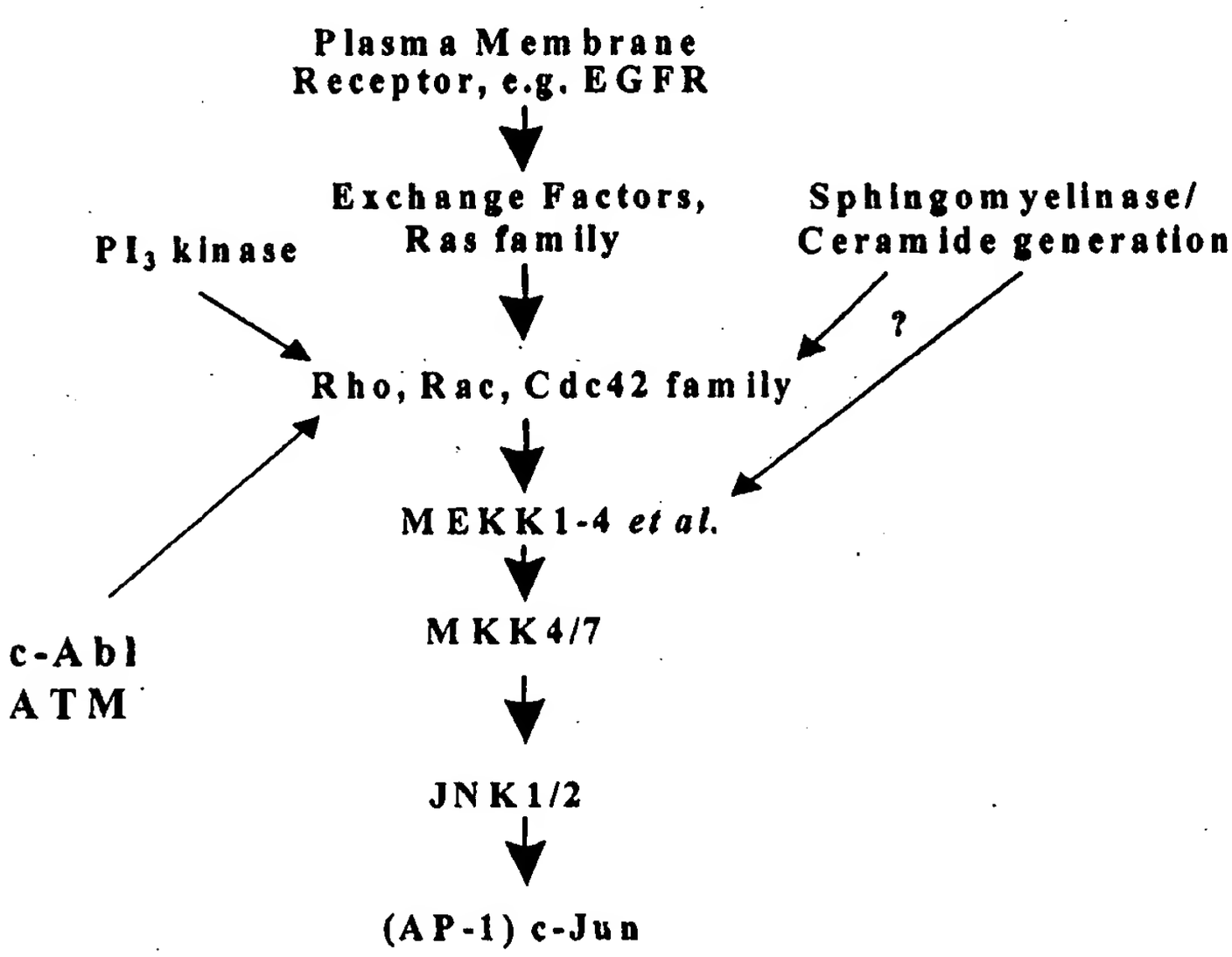

Figure 2. The JNK pathway in mammalian cells.

3. AN OVERVIEW OF THE ROLE OF THE MAPK PATHWAY IN PROLIFERATION, DIFFERENTIATION AND SURVIVAL SIGNALING

Simplistically, cell growth can be divided into five separate phases termed the cell cycle. Quiescent, non-proliferating cells are frequently termed to be in G_0 phase. Upon mitogenic stimulation, cells in G_0 enter into the first growth phase of the cell cycle, G_1. Once cell growth has reached a certain level, cells enter a new phase of the cycle as they begin to synthesize new DNA, which is termed S phase. Cells exit S phase and enter a shorter growth phase termed G_2, which is shortly followed by chromosomal alignment along the metaphase plate as cells enter into M (mitosis) phase. The chromosomes separate in M phase and two daughter cells are formed. Both the MAPK and JNK pathways have been proposed to control cell cycle progression.

Initial observations suggested that signaling by the MAPK pathway was intimately involved in the abilities of growth factors to stimulate proliferation and initially the accepted view of signaling through the pathway was that its activation promotes proliferation, and the greater the activation, the greater the proliferative response[56]. For example, in NIH 3T3 fibroblasts, transformation with either the v-Ha-Ras oncogene or the v-Raf oncogene caused constitutive activation of the MAPK cascade and increased proliferation[57,58]. In NIH 3T3 cells, expression of a constitutively active form of MKK1 also caused constitutive activation of the MAPK cascade and increased proliferation[59]. The positive role of MAPK activation in cell cycle progression may be linked to increased expression of cyclin molecules, e.g., cyclin D1[60–63]. However, more defined studies examining the extent and duration of MAPK activation are now beginning to show that a simplistic view of increased activation of MAPK equating with increased proliferation is not necessarily valid.

For example, in PC12 pheochromocytoma cells, the role of MAPK signaling appears to conflict with the conventional view linking increased activity to enhanced proliferation. It was known that exposure of PC12 cells to EGF stimulates their proliferation. In contrast, exposure of these cells to nerve growth factor (NGF) was shown to inhibit proliferation and cause a differentiation response[64,65]. Several groups then noted that whereas EGF induces an acute phasic activation of the MAPK pathway, NGF increases MAPK activity over a prolonged time period[66–68]. Other studies confirmed that prolonged signaling, via the MAPK pathway, was essential to the ability of NGF to induce differentiation. It was argued[68,69] that the ability of NGF to cause growth arrest via MAPK is dependent upon its ability to increase expression of the cyclin dependent kinase inhibitor protein (CKI) $p21^{Cip\text{-}1/MDA6/WAF1}$. Many leukemic cell types behave in a similar manner to PC12 cells when exposed to phorbol esters. For example, Whalen *et al.*[70] demonstrated that phorbol esters cause growth arrest and differentiation in megakaryocytes via activation of classical PKC isoforms. These enzymes cause prolonged activation of the MAPK pathway, leading to terminal

differentiation[70]. Exposure of myeloid leukemia cells to phorbol esters can also increase $p21^{Cip\text{-}1/MDA6/WAF1}$ expression via the MAPK pathway[71].

Recently, studies performed in embryonic fibroblasts and in primary hepatocytes have demonstrated definitively that an acute phasic activation of the MAPK pathway promotes proliferation, whereas prolonged activation of the pathway promotes cell cycle arrest[72–75]. In studies by McMahon and Land, mouse embryonic fibroblasts which were p21 -/- did not arrest in response to prolonged MAPK activation, suggesting a key role for $p21^{Cip\text{-}1/MDA6/WAF1}$ in the MAPK-mediated cell cycle arrest[72,73]. In studies by Park *et al.*[75] and Tombes *et al.*[74], however, prolonged MAPK signaling was observed to increase expression of both $p21^{Cip\text{-}1/MDA6/WAF1}$ and another CKI protein, $p16^{INK4a}$, in primary hepatocytes. This suggests that MAPK can modify expression of different cassettes of CKI proteins in a cell-type specific manner, which may in turn exert cell type specific functions in mediating cell growth arrest.

However, it is notable that inhibition of PKC function in megakaryocytes promotes differentiation towards an erythroid lineage, suggesting that perturbations in PKC signaling, potentially via downstream recruitment of MAPK, can lead to a switch between specific differentiation pathways[76]. Similarly, other studies have suggested that increased or decreased MAPK signaling can influence T cell differentiation between either a CD4 or CD8 expressing cell lineage[77]. In other cell types, several groups have demonstrated that MAPK signaling can both promote and inhibit adipogenesis and myogenesis in pre-adipocytes and myoblasts, respectively, in a time and context dependent manner[78–81]. Thus in some established cell systems, *constitutive elevation* of MAPK activity can stimulate proliferation, whereas in others it triggers increased $p21^{Cip\text{-}1/MDA6/WAF1}$ levels, cell cycle arrest, and cellular maturation. In contrast, in other cell types, *prolonged inhibition* of the MAPK pathway may also promote maturation and lead to increased $p21^{Cip\text{-}1/MDA6/WAF1}$ expression. The linkage of MAPK signaling to regulation of $p21^{Cip\text{-}1/MDA6/WAF1}$ expression is potentially important from a therapeutic perspective. For example several studies have shown that p21 -/- cells or cells expressing p21 antisense have increased chemo- and radio-sensitivities[82–83], suggesting that the relative ability of a cell to express $p21^{Cip\text{-}1/MDA6/WAF1}$ will alter its responsiveness to a variety of cytotoxic cellular stresses. One implication from these studies is that a potential strategy to sensitize cells to ionizing radiation or chemotherapeutic agents may involve inhibition of the MAPK pathway. Other protective mechanisms may also exist. For example, recent studies suggest that MAPK may be involved in phosphorylation of Bcl-2 which, at least under certain conditions, may exert an anti-apoptotic effect[84]. Other groups have argued that protein levels of the anti-apoptotic protein Mcl-1 are regulated by MAPK signaling, potentially linking MAPK signaling to expression of an anti-apoptotic effector[85].

4. AN OVERVIEW OF THE ROLE OF THE JNK PATHWAY IN PROLIFERATION, DIFFERENTIATION AND APOPTOTIC SIGNALING

Since the JNK pathway was first examined as a pathway activated in response to cytotoxic insults, many of the initial studies on JNK signaling focused on the role of this pathway as either a pro-apoptotic or anti-apoptotic effector. For example, Verheij *et al.*[86] demonstrated that exposure of U937 leukemic cells to either TNFα, FAS-ligand, ceramide or γ-radiation activated the JNK pathway and that this activation was causal in an enhanced apoptotic response to these stress signals. Multiple other studies over the past 10 years, using a large variety of cytotoxic stresses, have made similar conclusions; that JNK activation is a causal effector in pro-apoptotic signaling. These conclusions were strongly supported by molecular inhibition of the JNK pathway at multiple levels by expressing dominant negative versions of MKK4/7, JNK1/2 and c-Jun[87].

However, the multiple mechanisms by which prolonged JNK signaling can cause apoptosis in response to many stimuli are still not fully elucidated. One mechanism may be by the induction of death receptors and/or their ligands, such as the APO-1/CD95/FAS-Receptor and FAS-ligand[88]. Alternatively, JNK has been proposed to phosphorylate anti-apoptotic mitochondrial proteins, e.g., Bcl-xl, causing inactivation of Bcl-xl anti-apoptotic function, thereby also promoting apoptosis[89]. Other studies have shown that while JNK signaling may play a role in apoptosis, it does not necessarily play an *initiating* role. For example, some studies have suggested that while cytotoxic drugs can activate apoptotic caspase proteases, a profound apoptotic response after drug exposure requires an additional proteolytic cleavage of MEKK enzymes followed by JNK activation[90]. In contrast, Herr *et al.*[91] demonstrated that apoptotic caspase activation did not correlate with JNK activation in cells treated with doxorubicin. In MCF-7 mammary carcinoma cells, exposure to TNFα caused JNK activation in both TNFα-sensitive and -insensitive cell lines, but only caused apoptosis in the sensitive cell line variant[92]. In part, this effect may be because the resistant cell line had a lower activation of JNK, further suggesting that the time and amplitude of pathway activation plays a key role in the cellular response to any pathway. Behrens *et al.*[93], using primary fibroblasts from transgenic mice expressing a c-Jun mutant mutated at the NH_2-terminal phosphorylation sites, found that loss of these sites impaired both proliferative and apoptotic responses of these cells. In an analogous manner to our comments surrounding MAPK signaling, these findings have shed doubt upon the concept that JNK signaling is obligatorily required for apoptosis.

Several groups have demonstrated that JNK signaling can represent an important pro-proliferative or differentiation signal in a variety of cells. As noted above a mutant c-Jun, which could no longer be phosphorylated by JNK, impaired fibroblast growth. In A549 cells loss of JNK function abolished EGF-stimulation of growth[94]; similar data have been obtained in primary hepatocytes stimulated with TNFα or hepatocyte growth factor[95]. In

contrast, JNK signaling also has been linked to a differentiation response in other cells such as pre-erythrocytes and pre-T helper cells[96,97]. Thus it has been proposed that JNK signaling may either cause growth/differentiation or death in a contextual manner. For example, a short burst of high JNK activity or low sustained JNK activity may cause a growth/differentiation response whereas high sustained JNK activity, potentially after cleavage of upstream activators such as MEKK, may lead to an apoptotic response.

5. POTENTIAL DIRECT ROLES FOR MAPK AND JNK SIGNALING IN THE CONTROL OF THE CELL CYCLE AND DNA REPAIR FOLLOWING IRRADIATION AND DRUG EXPOSURE

Evidence is now emerging that the MAPK pathway can play both positive and negative roles in cell survival after treatment with various chemotherapeutic drugs and/or ionizing radiation[98–101]. More recently, other studies using cytotoxic drugs have also surprisingly suggested that enhanced JNK signaling can enhance cell survival by increasing DNA repair.

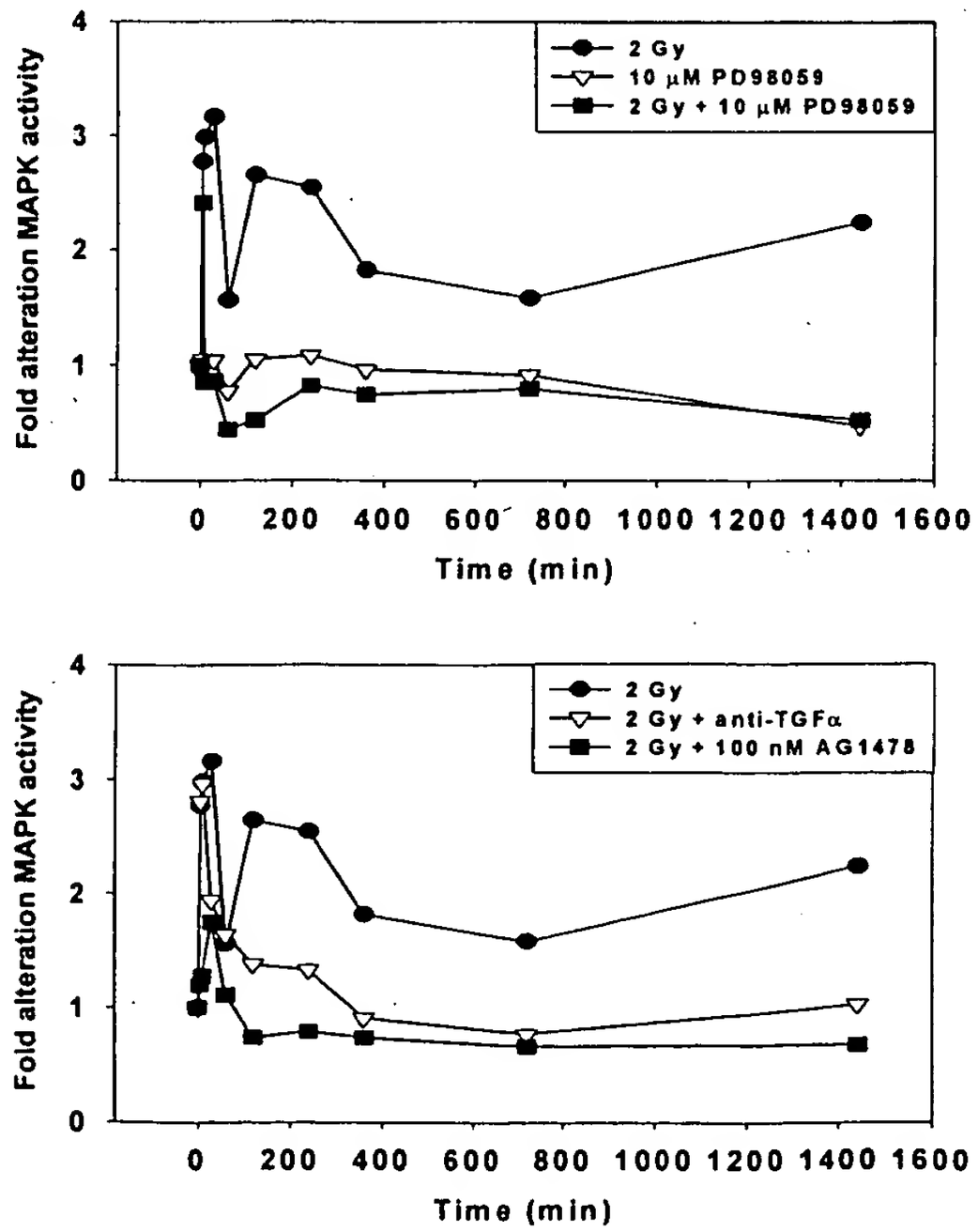

Figure 3. Ionizing radiation activates MAPK in DU145 prostate carcinoma cells via the EGFR and TGFα.

6. MAPK SIGNALING

Several studies have suggested both a radio-protective and a radio-sensitizing role for MAPK signaling. In both instances, the differential effects appear to be due to altered cell cycle progression following radiation exposure. Radiation can increase MAPK activity in a variety of cell types by causing activation of growth factor receptors in the plasma membrane[102]. One component of this activation may be operating through the actions of autocrine growth factors such as TGFα[103] (Figure 3). This may be important because tumor cells tend to express more receptors and more autocrine ligand than non-transformed cells, thus leading to higher activities and activation of protective signal transduction pathway(s) such as MAPK[104].

Previous studies have shown that activation of PKC by either phorbol esters or bryostatin 1 shortly before or after irradiation exerts cytoprotective effects toward normal human hematopoietic progenitor cells *in vitro* and in intact animals *in vivo*[105]. However, down-regulation of PKC expression by a 24 h pre-treatment with bryostatin 1 was found to sensitize cells to ionizing radiation[106]. Downstream inhibition of the MAPK pathway by the selective MEK1/2 inhibitor PD98059 also increases the radiosensitivity of cells and causes a prolonged G_2/M arrest[103,107]. Cells which are arrested in G_2/M then undergo apoptosis, and the remaining non-apoptotic cells display a loss of clonogenicity in clonogenic assays[103,107,108], in general agreement with the capacity of PKC down-regulation to exert a similar effect[106]. Thus, interruption of PKC signaling, or one of its downstream targets such as the MAPK pathway, can lower the apoptotic threshold of cells in response to ionizing radiation and certain chemotherapeutic agents.

Increased expression of Raf-1 has been shown to both radio-sensitize[109] and to enhance radio-resistance[110]. The radio-sensitizing effect of enhanced Raf-1 expression was correlated with a more rapid exit from radiation-induced G_2/M arrest and an enhanced G_1/S arrest. Other studies, however, have argued that enforced over-expression of Raf-1 is radio-protective and that ablation of Raf-1 expression by use of antisense oligonucleotides radio-sensitizes these cells[111]. In both instances it should be noted that enhanced Raf-1 protein levels may not cause a large increase in basal MAPK activity, but could enhance radiation-induced stimulation of the MAPK pathway. If enhanced Raf-1 protein levels do increase MAPK signaling, it is possible that expression of CKI molecules such as $p21^{Cip\text{-}1/WAF1/mda6}$ and $p16^{INK4a}$ may be induced, leading to a prolongation of growth arrest at both G_1/S and potentially at G_2/M phases[74,75]. Depending upon the cell type and the extent of CKI induction, it may be possible to observe these effects only at the G_1/S transition or at both G_1/S and G_2/M. This effect will in turn alter the cell cycle profile after irradiation.

In general agreement with a role for MAPK signaling in enhancing G_2/M progression, other groups have shown that inhibition of MAPK signaling can prolong radiation-induced G_2/M arrest and increase radio-sensitivity[103,107,108]. The role of the MAPK pathway in cell cycle progression at the G_2/M transition has been found to be complex, and it appears that MAPK signaling is both permissive for G_2/M entry and G_2/M exit[112,113]. These observations suggest that a certain amount of radiation-induced MAPK activity may

enhance progression through G_2/M phase, increasing radio-sensitivity. In agreement with this notion, either enhanced Raf-1 expression or pre-treatment of cells with caffeine can reduce radiation-induced G_2/M arrest and radiosensitize cells. However, in studies by Abbott and Holt[107], and by Park *et al.*[108], addition of caffeine several hours after exposure abolished the prolonged portion of the G_2/M arrest caused by MAPK inhibition and was shown to be radio-protective. Thus it appears that either potentiation or inhibition of radiation-induced G_2/M arrest can decrease cell survival after exposure to ionizing radiation.

After exposure to ionizing radiation, cells arrest their growth so as to repair their DNA[114], and the time spent in growth arrest at G_2/M, DNA repair and radio-sensitivity have been correlated in several studies[115]. In the study by Abbott and Holt, no effect of MAPK inhibition was observed on DNA repair after radiation exposure[107]. However MAPK signaling has been linked to expression of at least one protein involved in nucleotide excision repair, ERCC1[116]. Under normal culture pO_2 conditions, loss of ERCC1 expression does not significantly alter radiosensitivity whereas under hypoxic conditions, loss of ERCC1 was recently found to reduce survival after irradiation[117,118] Thus MAPK signaling may be able to modify the DNA repair response of some cells under certain conditions.

Additional insights into the potential roles of MAPK in the regulation of drug-mediated lethality have arisen from studies involving the nucleoside analog ara-C. Ara-C is converted in cells to its active triphosphate derivative, ara-CTP, which is incorporated into elongating strands of DNA, resulting in interference with chain elongation and promotion of chain termination[119]. Exposure of cells to ara-C causes generation of lipid messengers that exert opposing effects on cell survival; ceramide and diglyceride. This raises the possibility that the relative extent to which these signaling molecules are generated determines the cell's ultimate fate. Furthermore, the cytoprotective effects of MAPK may exert a self-limiting effect on ara-C-mediated lethality. It would be predicted that interventions that reduce net MAPK activity would potentiate ara-C-related cytotoxicity. In agreement with this hypothesis, we and others have found that pharmacologic agents which directly inhibit PKC (e.g., staurosporine)[120], down-regulate PKC upon chronic exposure (e.g., bryostatin 1), or ablate PKC expression (e.g., PKC antisense oligonucleotides) have all been shown to enhance the lethal actions of ara-C[121–123]. Interestingly, in the latter study, ceramide exposure decreased whereas diglyceride exposure increased expression of the anti-apoptotic protein Bcl-2 in leukemic cells[123,124]. More recently, bryostatin 1 and the PKC inhibitor safingol have been shown to block ara-C-mediated MAPK activation in association with potentiation of leukemic cell apoptosis, effects which we have found to be mimicked by the MEK1 inhibitor PD98059[121,125,126]. Potentiation of ara-C-mediated lethality by inhibition of the MAPK pathway has also been observed in PC12 cells[127]. In contrast, others have shown that loss of MAPK function did not enhance ara-C cell killing[128]. Clearly, the ability of MAPK to protect cells from ara-C-induced DNA damage may be a cell-type dependent effect. Collectively, these findings raise the possibility that interruption of the PKC/MAPK pathway facilitates apoptosis, perhaps by preventing one or more cytoprotective responses.

Finally, attention has also been focused on alternate downstream MAPK cytoprotective effectors. These include the transcription factors NFκB[129] and CREB[130], which have recently been shown to protect cells from growth factor deprivation-induced apoptosis in cells of a neural origin. Whether these effectors contribute to the cytoprotective effects of the MAPK cascade in cells exposed to radiation or drugs remains to be determined.

7. JNK SIGNALING

As previously discussed, inhibition of PKC and/or MAPK function radiosensitizes fibroblast and epithelial cell types, and conversely, PKC activation has been shown to attenuate ionizing radiation-mediated lethality[131,132]. The lethal effects of ionizing radiation in some cell types, e.g., in peripheral lymphoblasts from individuals with Neimann-Pick disease, has been directly attributed to the cytotoxic actions of ceramide[133]. The ability of radiation to activate the JNK pathway has been investigated in leukemia cells and demonstrated to be dependent upon activation of sphingomyelinase enzymes[134]. These data suggest that sphingomyelinase enzymes, via the JNK pathway, play key roles in mediating radiation-induced cytotoxic signals.

However, based on studies using growth factors and non-lymphoid cell types, many other stimuli could play a role in JNK activation following irradiation. For example, in carcinoma cells radiation-induced signaling from TGFα and the EGFR, and the TNFα receptor, can play a role in JNK activation[103]. Of note, many ligands utilize signaling through the cytoprotective PI_3 kinase pathway to activate JNK, suggesting that JNK signaling under certain circumstances may be protective[53]. Other studies have argued that radiation-induced DNA damage may play a role in JNK activation by protein kinases such as ATM (mutated in ataxia telangiectasia) and c-Abl[135]. These observations raise the intriguing possibility that stress-induced JNK pathway activation may be under the control of multiple factors; receptor ligands; growth factor receptors; sphingomyelinase enzymes and ceramide; and DNA damage-activated protein kinases such as ATM and c-Abl.

Multiple studies have documented the cytotoxic effects of prolonged JNK signaling in a variety of cell types[93]. It is also clear that increased JNK signaling does not always correlate with increased apoptosis after exposure to a cytotoxic stress. For example, Reardon *et al.*[136] demonstrated that radiation-induced activation of JNK was not causal in the apoptotic response of MDA-MB-231 mammary carcinoma cells. This observation may be in part due to the fact that a parallel balanced activation of both JNK and MAPK signaling occurs in response to this stimulus.

It is also becoming evident that JNK signaling may play an important cytoprotective effector in the response of cells to DNA damage. For example, Potapova *et al.*[137] demonstrated that cells exposed to cisplatin were more sensitive to drug-induced apoptosis if their JNK signaling pathway was inhibited. In addition, in cells which were DNA repair deficient, Nehme *et*

al.[138] showed that no activation of c-Abl or JNK took place, leading to a more cisplatin-sensitive phenotype. Exposure of cells to UV radiation causes DNA damage and several investigators have argued that JNK pathway signaling is involved in the repair of UV-damaged DNA. Engelberg *et al.*[139] and Schreiber *et al.*[140] have both shown data arguing that the AP-1 (c-Jun:c-Fos) transcription factor complex is an essential component in the protective response of cells exposed to UV radiation. Since c-Fos expression, which is under control of the transcription factor Elk-1, is dependent upon both MAPK and JNK signaling, it is likely that both JNK and MAPK signaling play roles in the protective response to UV radiation.

In the previous section examining MAPK signaling and DNA repair, it was proposed that MAPK regulates the ERCC1 promoter[116]. Other groups have shown that phorbol esters can increase ERCC1 promoter activity through an AP-1 responsive element, suggesting that signaling by the JNK pathway may also enhance ERCC1 expression[141,142]. Exposure of cells to nitrosoureas has been shown to induce cytotoxic DNA lesions by alkylation of guanine residues, and these lesions are repaired by O^6-methylguanine-DNA methyltransferase (MGMT) enzymes. The MGMT promoter contains two AP-1 binding sites and its activity increased after exposure to phorbol esters, suggesting a role for JNK/c-Jun signaling in this process[143]. Thus it is probable that both JNK and MAPK signaling will impact on c-Jun and c-Fos protein levels, respectively, and ultimately the function of the AP-1 complex leading to enhanced expression of multiple proteins involved in DNA repair processes.

CONCLUSION

From the previous sections it can be deduced that signaling by the MAPK and JNK pathways can control many of the responses of cells following exposure to toxic agents. Signaling by the MAPK pathway has been largely associated with cytoprotective responses whereas signaling by the JNK pathway has been associated with cytotoxic responses. However, although evidence in some cell systems indicates that JNK signaling can protect cells from death, in others it appears that JNK/AP-1 function is essential for the expression of certain DNA repair enzymes. Thus it seems likely that moderate levels of JNK activity may be required for protective responses of cells to DNA damage. In other systems, increased MAPK activity may enhance expression of cytotoxic ligands potentially leading to cell death, whereas in others it could enhance expression of anti-apoptotic proteins, leading to a protective response. The overall balance of protective and toxic signals generated by each pathway will ultimately depend upon the cell type examined and the culture conditions used. Currently, relatively little is known about the mechanism(s) by which signaling pathways control the expression and function of DNA repair genes. Further studies will thus be required to link enhanced signal transduction pathway function with enhanced DNA repair following exposure to DNA damaging agents.

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UNTANGLING THE ErbB SIGNALLING NETWORK

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When epidermal growth factor and its relatives bind the ErbB family of receptors, they trigger a rich network of signalling pathways, culminating in responses ranging from cell division to death, motility to adhesion. The network is often dysregulated in cancer and lends credence to the mantra that molecular understanding yields clinical benefit: over 25,000 women with breast cancer have now been treated with trastuzumab (Herceptin®), a recombinant antibody designed to block the receptor ErbB2. Likewise, small-molecule enzyme inhibitors and monoclonal antibodies to ErbB1 are in advanced phases of clinical testing. What can this pathway teach us about translating basic science into clinical use?

MESENCHYME
Immature connective tissue that consists of cells embedded in extracellular matrix.

NEUREGULINS
EGF-like ligands whose primary receptor is ErbB3 and/or ErbB4. Four types of neuregulin are known.

STROMA
Supporting connective tissue in which a glandular or other epithelium is embedded.

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ErbBs are typical receptor tyrosine kinases that were implicated in cancer in the early 1980s when the avian erythroblastosis tumour virus was found to encode an aberrant form of the human epidermal growth factor (EGF) receptor (also known as ErbB1, HER or EGFR). Since then, the ErbB family has grown to four, and we are beginning to appreciate that the normal function of ErbBs and their ligands is to mediate cell–cell interactions in organogenesis and adulthood (reviewed in REF. 1).

In the epithelium, the basolateral location of ErbBs enables them to mediate signals between the MESENCHYME and the epithelium for cell growth[2]. The mesenchyme serves as a storehouse for many ligands including NEUREGULINS (NRGs), which bind ErbB3 and ErbB4. ErbB2 (also known as HER2) is a more potent oncoprotein than the other ErbBs, but no known ligand binds it with high affinity. It was first discovered as a rodent carcinogen-induced oncogene that encodes a variant of ErbB2 with a mutation that makes its tyrosine kinase constitutively active. ErbB2 is a shared co-receptor for several STROMAL ligands. Blocking the action of ErbB2 might thus inhibit a myriad of mitogenic pathways affecting ErbB-expressing tumour cells[3]. Although several strategies are being developed, Herceptin® — a HUMANIZED MONOCLONAL ANTIBODY to ErbB2 — has been the first to reach widespread clinical use, in particular for the treatment of metastatic breast cancer[4,5].

A layered signalling network

The components of the ErbB signalling pathway are evolutionarily ancient (BOX 1), and at first glance resemble a simple growth factor signalling pathway: ligand binding to a monomeric receptor tyrosine kinase activates the cytoplasmic catalytic function by promoting receptor dimerization and self-phosphorylation on tyrosine residues. The latter serve as docking sites for various ADAPTOR PROTEINS or enzymes, which simultaneously initiate many signalling cascades to produce a physiological outcome (FIG. 1). In higher eukaryotes, the simple linear pathway has evolved into a richly interactive, multilayered network, in which combinatorial expression and activation of components permits context-specific biological responses throughout development and adulthood.

The input layer. This comprises the ligands (EGF family of growth factors) and their receptors — the ErbBs (FIG. 1). All high-affinity ErbB ligands have an EGF-LIKE DOMAIN and three disulphide-bonded intramolecular loops. This receptor-binding domain is usually part of a large transmembrane precursor containing other structural motifs such as IMMUNOGLOBULIN-LIKE DOMAINS, heparin-binding sites and glycosylated linkers. Expression and processing of the precursor are highly regulated. For example, transformation by active Ras, or exposure to steroid hormones[6] leads to increased expres-

Box 1 | Evolution of the ErbB signalling network

Both the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* have primordial linear versions of the ErbB signalling pathway. In higher organisms, this has evolved into a complex network, probably because an interconnected layered structure can confer selective gains in terms of adaptation, tolerance to mutations and signal diversification[91]. The main functional features of the ErbB module were defined in invertebrates: ErbB regulates the fate of diverse cell lineages in different developmental stages through short-range paracrine interactions.

C. elegans and *Drosophila* each contain a single ErbB homologue; however, the only EGF-like ligand of *C. elegans*, called Lin-3, is replaced by four ligands in *Drosophila*. Vulva development is a well-characterized function of the Lin-3 signalling pathway: the six vulva precursor cells (VPCs) respond to an inductive signal from a gonadal anchor cell, which is thought to secrete Lin-3. Lin-3 binds the juxtaposed receptor on one of the VPCs and instructs it to undergo several cell cycles and develop concomitantly a more differentiated phenotype. The Lin-3 pathway functions in other inductive morphogenic events; loss-of-function mutations in the receptor result not only in a vulvaless phenotype, but also in sterility, abnormal male tail development and death[92].

The *Drosophila* EGF receptor (DER) is used repeatedly in several stages of development, including oogenesis, embryogenesis, and wing and eye development. Likewise, differentiation of the DER-expressing tendon cell is regulated by the myotube-derived NRG-like ligand, Vein[93]. Gurken, a homologue of transforming growth factor-α (TGF-α), functions primarily in the oocyte. Activation of another ligand, Spitz, which is anchored to the cell surface, requires proteolytic cleavage[94]. By contrast, Argos, a secreted DER ligand, is unique in that it negatively acts on receptor signalling[95].

sion of several ErbB ligands, and cleavage of ligand precursors by a METALLOPROTEINASE can be stimulated by activation of other receptors, such as G-protein-coupled receptors[7] (FIG. 2).

An important issue relates to the multiplicity and possible redundancy of ErbB ligands. This issue is particularly relevant to the many NRGs and their splice variants. Studies in cultured cells and initial attempts to address this issue in animals suggest that ErbB ligands have non-overlapping functions. For example, ligands such as EGF and NRG4, which bind to ErbB1 and ErbB4, respectively, have narrow specificity, whereas others such as epiregulin, NRG1β and betacellulin bind to two distinct primary receptors[8]. Overexpression of ErbB2, which biases heterodimer formation, can broaden ligand specificity (FIG. 1, dotted lines), and ligands that are better at recruiting this co-receptor can reduce the binding of less effective ligands. In addition, splice variants of NRGs and various ligand–receptor complexes also differ in their ability to recruit a partner receptor[9–11], which affects their potency and kinetics of signalling.

The four ErbBs share an overall structure of two cysteine-rich regions in their extracellular region, and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites. With few exceptions (for example, haematopoietic cells), ErbB proteins are expressed in cells of MESODERMAL and ECTODERMAL origins.

Examination of the intracellular and extracellular domains of the ErbBs provides a satisfying explanation as to why a horizontal network of interactions is crucial to the ErbB signalling pathway: ErbB3 is devoid of intrinsic kinase activity[12], whereas ErbB2 seems to have no direct ligand[13]. Therefore, in isolation neither ErbB2 nor ErbB3 can support linear signalling (FIG. 3). Most inter-receptor interactions are mediated by ligands, and ErbB2-containing heterodimers are formed preferentially[14,15]. Nevertheless, overexpression of a specific receptor can bias dimer formation, especially in the case of ErbB2, whose homodimers can spontaneously form in ErbB2-overexpressing cells. Many cancers of epithelial origin have an amplification of the ErbB2 gene, which pushes the equilibrium towards ErbB2 homodimer and heterodimer formation. By contrast, ErbB4, whose expression pattern is relatively limited, has several isoforms that differ in their juxtamembrane and carboxyl termini, resulting in differences in the recruitment of phosphatidylinositol-3-OH kinase (PI(3)K)[16], which activates cell-survival pathways.

Signal-processing layers. The specificity and potency of intracellular signals are determined by positive and negative effectors of ErbB proteins, as well as by the identity of the ligand, oligomer composition and specific structural determinants of the receptors. The main determinant, however, is the vast array of phosphotyrosine-binding proteins that associate with the tail of each ErbB molecule after engagement into dimeric complexes (FIG. 1). Which sites are autophosphorylated, and hence which signalling proteins are engaged, are determined by the identity of the ligand as well as by the heterodimer partner[17]. The Ras- and Shc-activated mitogen-activated protein kinase (MAPK) pathway is an invariable target of all ErbB ligands, and the PI(3)K-activated AKT PATHWAY and p70S6K/p85S6K pathway are downstream of most active ErbB dimers. The potency and kinetics of PI(3)K activation differ, however, probably because PI(3)K couples directly with ErbB3 and ErbB4, but indirectly with ErbB1 and ErbB2 (REF. 18).

Simultaneous activation of linear cascades, such as the MAPK pathway, the STRESS-ACTIVATED PROTEIN KINASE cascade, protein kinase C (PKC) and the Akt pathway translates in the nucleus into distinct transcriptional programmes. These involve not only the proto-oncogenes *fos*, *jun* and *myc*, but also a family of zinc-finger-containing transcription factors that includes Sp1 and Egr1, as well as Ets family members such as GA-binding protein (GABP)[19]. Despite sharing some pathways, each receptor is coupled with a distinct set of signalling proteins. For example, unlike ErbB1, the kinase-defective ErbB3 cannot interact with the adaptor protein and UBIQUITIN LIGASE c-Cbl, the adaptor protein Grb2, the second-messenger-generating enzyme phospholipase Cγ or the Ras-specific GTPase-activating protein (GAP)[20], but it can associate with the adaptors Shc and Grb7 (FIG. 1).In addition to combinatorial interactions, an important determinant of signalling outcome is variation in the kinetics of specific pathways. The principal process that turns off signalling by the ErbB network is ligand-mediated receptor endocytosis, and the kinetics of this process also depend heavily on receptor composition (BOX 2).

The output layer. The output of the ErbB network ranges from cell division and migration (both associated with tumorigenesis) to adhesion, differentiation and

HUMANIZED MONOCLONAL ANTIBODY
An antibody, usually from a rodent, engineered to contain mainly human sequences. This process reduces the immune response to the antibody in humans.

ADAPTOR PROTEINS
Proteins that augment cellular responses by recruiting other proteins to a complex. They usually contain several protein–protein interaction domains.

EGF-LIKE DOMAIN
A motif with ~50 amino acids, including six cysteine residues and a mainly β-sheet structure, found in all ErbB-binding growth factors and in extracellular matrix proteins.

IMMUNOGLOBULIN-LIKE DOMAIN
A protein domain composed of two β-pleated sheets held together by a disulphide bond.

METALLOPROTEINASES
Proteinases that have a metal ion at their active sites.

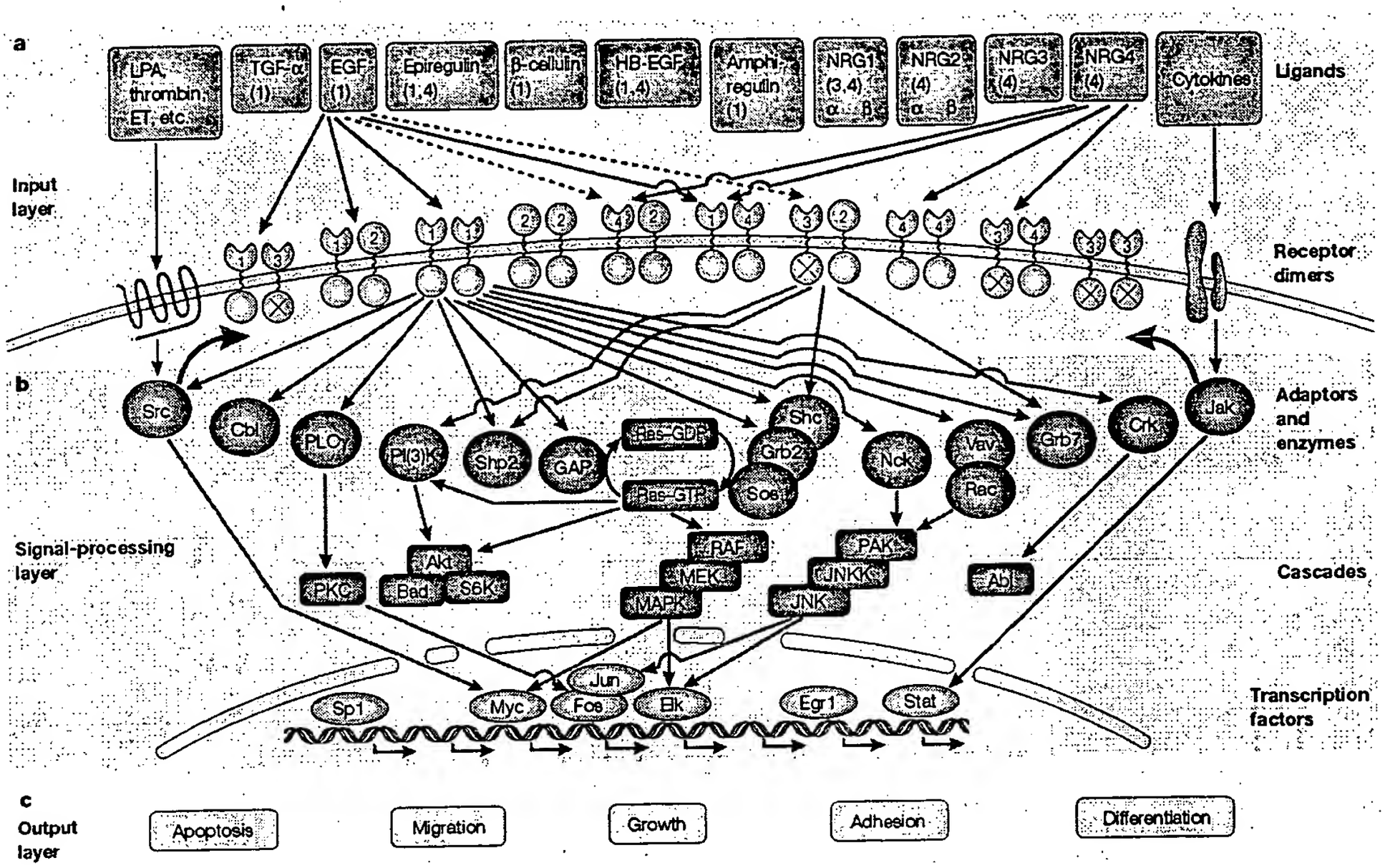


Figure 1 | **The ErbB signalling network. a** | Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors[8]. For simplicity, specificities of receptor binding are shown only for epidermal growth factor (EGF) and neuregulin 4 (NRG4). ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains). *Trans*-regulation by G-protein-coupled receptors (such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET)), and cytokine receptors is shown by wide arrows. **b** | Signalling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1 homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **c** | How they are translated to specific types of output is poorly understood at present. (Abl, a proto-oncogenic tyrosine kinase whose targets are poorly understood; Akt, a serine/threonine kinase that phosphorylates the anti-apoptotic protein Bad and the ribosomal S6 kinase (S6K); GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, janus kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription; RAF–MEK–MAPK and PAK–JNKK–JNK, two cascades of serine/threonine kinases that regulate the activity of a number of transcription factors.)

MESODERM
The middle germ layer of the developing embryo. It gives rise to the musculoskeletal, vascular and urinogenital systems, and to connective tissue (including that of the dermis).

ECTODERM
The outermost germ layer of the developing embryo. It gives rise to the epidermis and the nerves.

AKT PATHWAY
Akt (or protein kinase B) is a serine/threonine protein kinase activated by the phosphatidylinositol-3-OH kinase pathway that activates survival responses.

apoptosis (FIG. 1). Output depends on cellular context, as well as the specific ligand and ErbB dimer. This has been best shown in terms of mitogenic and transforming responses: homodimeric receptor combinations are less mitogenic and transforming than the corresponding heterodimeric combinations, and ErbB2-containing heterodimers are the most potent complexes[21–23] (FIG. 3).

Perhaps the best example of the ability of the ErbB module to tune mitogenic signalling is provided by the ErbB2–ErbB3 heterodimer: although neither ErbB2 nor ErbB3 alone can be activated by ligand, the heterodimer is the most transforming[24,25] and mitogenic[21] receptor complex. The ErbB2–ErbB3 heterodimer also increases cell motility on stimulation with a ligand[26]; but the other NRG receptor, ErbB4, which exists in several isoforms, has been associated with processes varying from cellular chemotaxis[27] to proliferation and differentiation[28].

A network of networks?

The ErbB network might integrate not only its own inputs but also heterologous signals, including hormones, neurotransmitters, lymphokines and stress inducers[29] (FIG. 1). Many of these *trans*-regulatory interactions are mediated by protein kinases that directly phosphorylate ErbBs, thereby affecting their kinase activity or endocytic transport[29]. The most extensively studied mechanism involves activation of G-protein-coupled receptors (GPCRs) by agonists such as lysophosphatidic acid (LPA), carbachol (which specifically activates muscarinic acetylcholine receptors) or thrombin (FIG. 2).

Experiments done with mutants and inhibitors of ErbBs imply that the mitogenic activity of some GPCR agonists requires transactivation of ErbB proteins. These agents increase tyrosine phosphorylation of ErbB1 and ErbB2, either by increasing their intrinsic

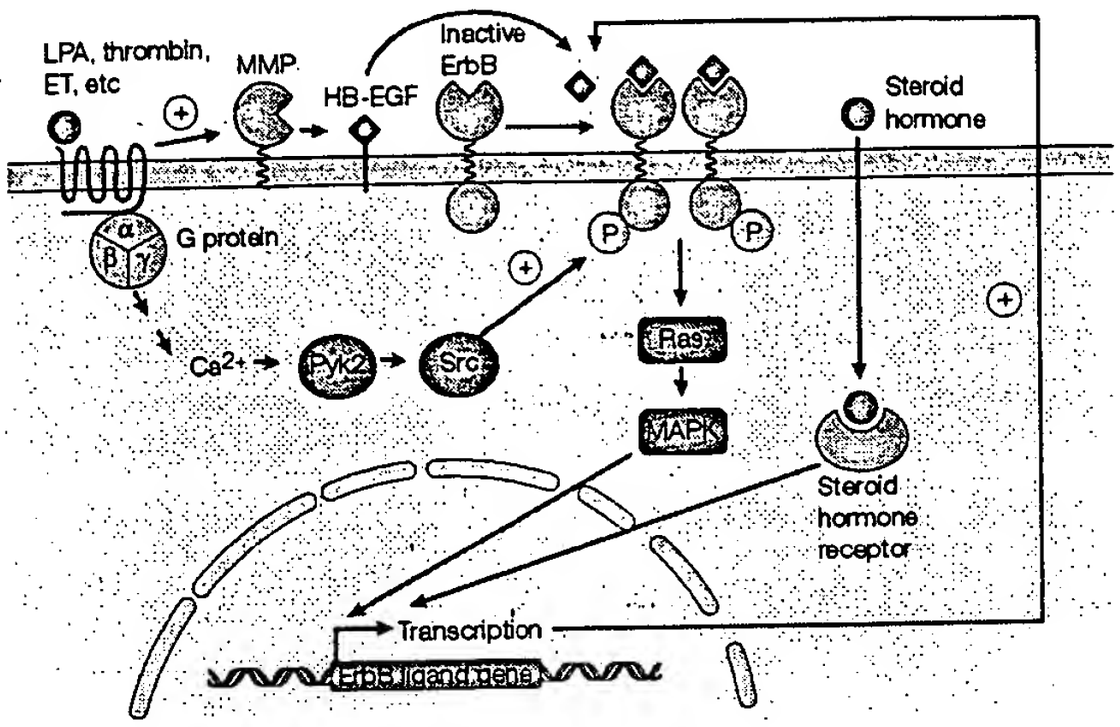


Figure 2 | **Crosstalk between the ErbB network and other signalling pathways.** G-protein-coupled receptors (GPCRs) such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET) can have positive effects on ErbB signalling through two mechanisms. First, through a poorly defined mechanism, they can activate matrix metalloproteinases (MMPs), which cleave membrane-tethered ErbB ligands (such as heparin-binding EGF-like factor, HB-EGF), thereby freeing them to bind to ErbBs. Second, GPCRs indirectly activate Src (perhaps via Pyk2), which phosphorylates the intracellular domains of ErbBs on tyrosine residues. Steroid hormones can have a positive effect on ErbB signalling by activating the transcription of genes encoding ErbB ligands. Finally, ErbB activation can activate a positive feedback loop through the Ras–MAPK (mitogen-activated protein kinase) pathway, which also activates transcription of ErbB ligand genes.

kinase activity[30] or by inhibiting an associated phosphatase activity. Signalling events downstream of ErbB1 are subsequently triggered, and this might account for the mitogenic potential of the heterologous agonists. Apparently, a cascade of tyrosine kinases links GPCRs such as the LPA receptor or the β-adrenergic receptor to ErbB1 and subsequently to MAPK. The cascade culminates in the stimulation of Src family kinases[31], which are recruited by either the calcium-regulated tyrosine kinase Pyk2 (REF. 32) or a GPCR-coupled kinase and an adaptor protein (for example, arrestin[33]). Another kinase that phosphorylates ErbB1 is the cytokine-regulated tyrosine kinase Jak2: on stimulation of adipocytes by growth hormone, Jak2 phosphorylates ErbB1, thus allowing MAPK activation even by a kinase-defective mutant of ErbB1 (REFS 34, 35).

Yet another cytokine, interleukin-6, elevates tyrosine phosphorylation of ErbB2 by increasing its intrinsic catalytic activity[36]. By contrast, factors that activate PKC, such as certain growth factors and hormones (for example, PDGF, LPA and EGF by itself), increase threonine and serine phosphorylation of ErbB1 and ErbB2, which decrease tyrosine phosphorylation and ligand binding affinity through a mechanism involving accelerated recycling of internalized receptors (BOX 2). These interconnections to other signalling modules help to integrate and coordinate cellular responses to extracellular stimuli.

STRESS-ACTIVATED PROTEIN KINASES
Members of the mitogen-activated protein kinase (MAPK) family that respond to stress. They include the Jun amino-terminal kinases (JNKs) and the p38 MAPKs.

UBIQUITIN LIGASES
Enzymes that catalyse the last stage of ubiquitylation, in which the small protein ubiquitin is transferred from a ubiquitin-conjugating enzyme (UBC or E2) to its target protein. They are also known as E3 enzymes.

GAPS
Proteins that inactivate small GTP-binding proteins, such as Ras family members, by increasing their rate of GTP hydrolysis.

Integrating developmental cues

The ErbB network is a key developmental signalling pathway throughout evolution. Its functions in worm and fly development are now well understood (BOX 1), but recent research using knockout and transgenic mice is beginning to clarify the functions of individual ErbBs and specific ligands in mammalian development.

ErbB1 and its ligands. Inactivation of ErbB1 impairs epithelial development in many organs, including those involved in tooth growth and eye opening[37–39]. Likewise, transgenic and *in vitro* studies implicate ErbB1 in promoting proliferation and differentiation of the epithelial component of skin, lung, pancreas and the gastrointestinal tract. These processes are probably regulated by growth factors from the local mesenchyme. Mice lacking expression of transforming growth factor-α (TGF-α) have abnormal skin, hair and eye development[40,41] but, in contrast with ErbB1-deficient mice, which undergo massive apoptosis in cortical and thalamic

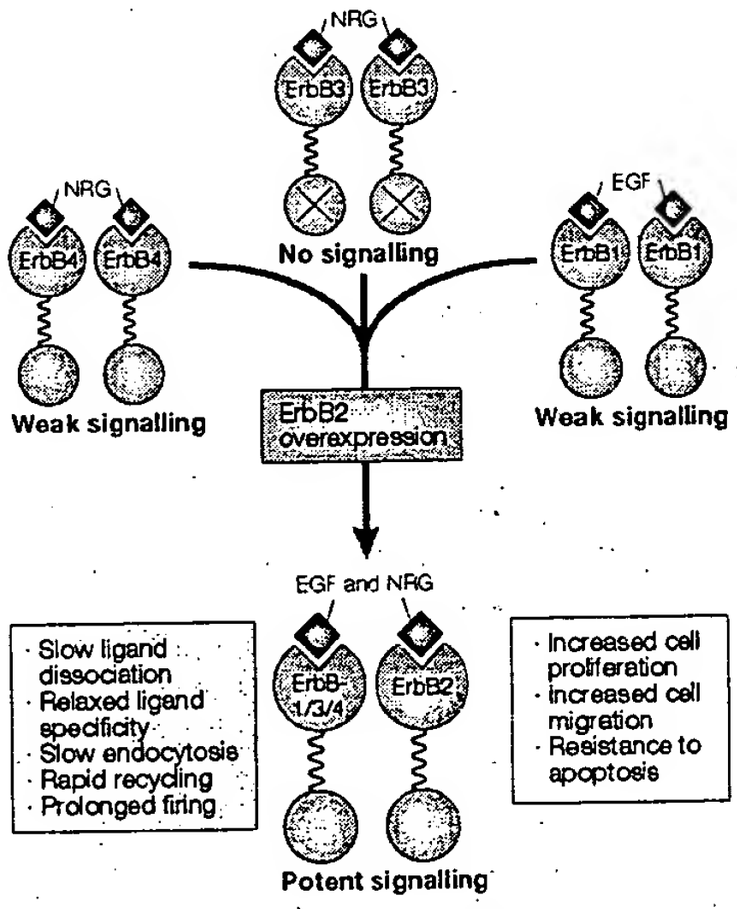


Figure 3 | **Signalling by ErbB homodimers in comparison with ErbB2-containing heterodimers.** Receptors are shown as two lobes connected by a transmembrane stretch. Binding of a ligand (EGF-like or NRG) to the extracellular lobe of ErbB1, ErbB3 (note inactive kinase, marked by a cross) or ErbB4 induces homodimer formation. When ErbB2 is overexpressed, heterodimers form preferentially. Unlike homodimers, which are either inactive (ErbB3 homodimers) or signal only weakly, ErbB2-containing heterodimers have attributes that prolong and enhance downstream signalling (green box) and their outputs (yellow box). Apparently, homodimers of ErbB2 are weaker signalling complexes than heterodimers containing ErbB2. (EGF, epidermal growth factor; NRG, neuregulin.)

Box 2 | Turning off the ErbB response

On ligand binding, ErbB1 molecules cluster over clathrin-coated regions of the plasma membrane, which invaginate to form endocytic vesicles. These mature to early and late endosomes, while gradually decreasing their internal pH and accumulating hydrolytic enzymes that lead to receptor degradation. Importantly, the other three ErbB proteins are endocytosis impaired and are more often recycled back to the cell surface[21,96]. Sorting to degradation is determined by the composition of the dimer: ErbB1 homodimers are targeted primarily to the lysosome; ErbB3 molecules are constitutively recycled[97]; and heterodimerization with ErbB2 decreases the rate of endocytosis and increases recycling of its partners[98,99]. Receptor internalization is determined by cytoplasmic motifs[100], but sorting in the early endosome seems to depend on the differential dissociation of ligand–ErbB complexes at mildly acidic pH. Complex dissociation leads to recycling, whereas continuous activation of tyrosine phosphorylation in the endosome leads to recruitment of c-Cbl, a ubiquitin ligase that preferentially binds to ErbB1 homodimers[101] and directs them to lysosomal degradation by tagging with polyubiquitin tracts[102].

brain regions[38], mice homozygous for a disrupted TGF-α gene show no brain abnormalities. So, the limited penetrance of TGF-α mutations and the confinement of the phenotype to the skin and eye suggest that each ErbB ligand has a distinct functional role and tissue specificity, analogous to the different roles played by each of the *Drosophila* EGF receptor ligands in insect development (BOX 2).

Neuregulins and their receptors. Like ErbB1 and its ligands involved in mesenchyme–epithelium interactions, the NRGs and their receptors are involved in the interaction between nerves and their target cells (for example, muscle, GLIA and SCHWANN CELLS), and are essential for cardiac and neural development. Mice defective in ErbB4, ErbB2 and NRG-1 die at embryonic day 10.5 from similar heart defects[1]. Endocardium-derived

Table 1 | **Expression of ErbBs and their ligands in cancer**

Molecule	Nature of dysregulation	Type of cancer	Notes	References
Ligands				
TGF-α	Overexpression	Prostate	Expressed by stroma in early, androgen-dependent prostate cancer and by tumours in advanced, androgen-independent cancer	52
	Overexpression	Pancreatic	Correlates with tumour size and decreased patient survival; may be due to overexpression of Ki-Ras, which also drives expression of HB-EGF and NRG1	108
	Overexpression	Lung, ovary, colon	Correlates with poor prognosis when co-expressed with ErbB1	51
NRG1	Overexpression	Mammary adeno-carcinomas	Necessary, but not sufficient for tumorigenesis in animal models	109
Receptors				
ErbB1	Overexpression	Head and neck, breast, bladder, prostate, kidney, non-small-cell lung cancer	Significant indicator for recurrence in operable breast tumours; associated with shorter disease-free and overall survival in advanced breast cancer; may serve as a prognostic marker for bladder, prostate, and non-small-cell lung cancers	110,111
	Overexpression	Glioma	Amplification occurs in 40% of gliomas; overexpression correlates with higher grade and reduced survival	35
	Mutation	Glioma, lung, ovary, breast	Deletion of part of the extracellular domain yields a constitutively active receptor	54
ErbB2	Overexpression	Breast, lung pancreas, colon oesophagus, endometrium, cervix	Overexpressed owing to gene amplification in 15–30% of invasive invasive ductal breast cancers Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors	56
ErbB3	Expression	Breast, colon gastric, prostate, other carcinomas	Co-expression of ErbB2 with ErbB1 or ErbB3 in breast cancer improves predicting power	64,65
	Overexpression	Oral squamous cell cancer	Overexpression correlates with lymph node involvement and patient survival	112
ErbB4	Reduced expression	Breast, prostate	Correlates with a differentiated phenotype	66
	Expression	Childhood medullo-blastoma	Co-expression with ErbB2 has a prognostic value	67

(TGF-α, transforming growth factor-α; NRG1, neuregulin-1; HB-EGF, heparin-binding epidermal growth factor.)

GLIA
Supporting cells of the nervous system, including oligodendrocytes and astrocytes in the central nervous system, and Schwann cells in the peripheral nervous system. Glia surround neurons, providing mechanical and physical support, and electrical insulation between neurons.

SCHWANN CELLS
Cells that produce myelin and ensheath axons in the peripheral nervous system.

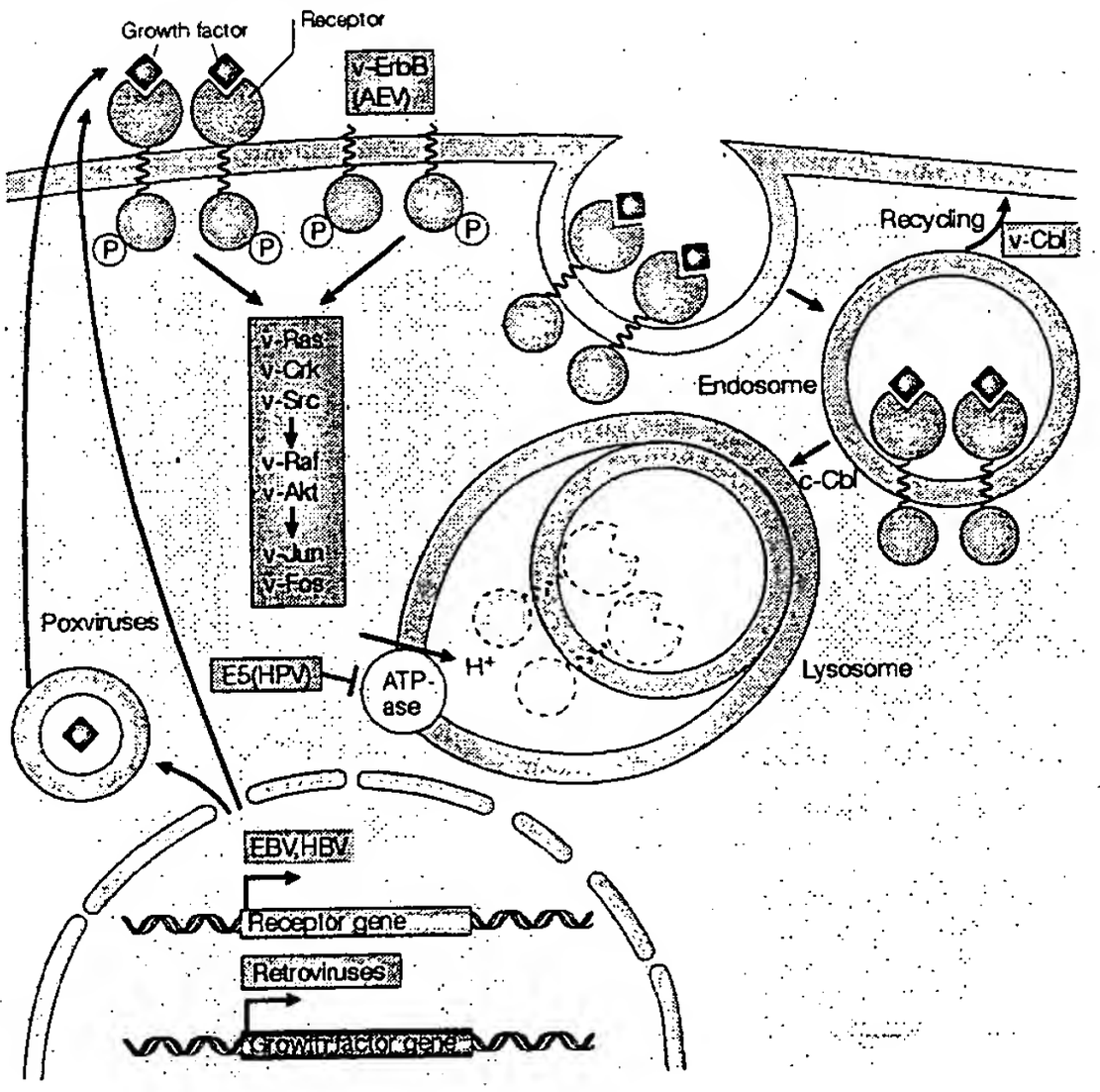
NRG1 stimulates an ErbB2–ErbB4 heterodimer on adjacent myocytes to initiate formation of the TRABECULAE. Surprisingly, the immunoglobulin domain and the cytoplasmic part of NRG1 — regions that are not involved in receptor binding — are essential for proper heart development[42,43]. ErbB3-deficient mice survive to embryonic day 13.5 and suffer from defective cardiac formation[44,45]. The alternative NRG-promoted heterodimer, ErbB2–ErbB3, is involved in different morphogenic events: mice lacking ErbB2, ErbB3 or NRG1 have a severely underdeveloped SYMPATHETIC GANGLION chain. This is probably caused by defective migration of neural progenitors from the NEURAL CREST[44].

The Schwann cell lineage is also controlled by the ErbB2–ErbB3 heterodimer. *In vitro* studies showed that NRG1 biases differentiation of neural crest progenitors towards a glial fate, and ErbB3-deficient mice showed partial lack of Schwann cells along peripheral and sensory neurons[45,46]. The ability of NRGs to control transcription of several ion channels underlies involvement of ErbBs in the neuromuscular junction[47]. NRGs elevate transcription of all subunits of the postsynaptic nicotinic acetylcholine receptor, but a nerve-derived splice variant seems to bias replacement of the γ-subunit with the ε-chain, which increases single-channel conductance. A similar subunit switch might occur at central synapses; NRG1β can markedly increase expression of the NR2C subunit of the *N*-methyl-D-aspartate receptor in slices of cerebellum[48].

The cancer connection

The potent cell proliferation signals generated by the ErbB network are used by cancer cells to fix oncogenic mutations by CLONAL EXPANSION. In addition, many types of oncogenic viruses exploit the ErbB network by manipulating its components (BOX 3). Human cancers use several mechanisms to activate the network at different layers. In many different cancer cell types, the ErbB pathway becomes hyperactivated by a range of mechanisms, including overproduction of ligands, overproduction of receptors, or constitutive activation of receptors (TABLE 1). It is extremely useful to know whether a

Box 3 | How do viruses harness the ErbB network?



Several transforming and non-transforming viruses constitutively elevate ErbB signalling by expressing an active component or by interfering with signalling shut-off. The hepatitis B virus (HBV), which is associated with hepatocellular carcinoma, upregulates transcription from the ErbB1 promoter[103]. Likewise, expression is deregulated by LMP1, a protein encoded by the Epstein–Barr virus (EBV), which is associated with several malignancies, including nasopharyngeal carcinoma[104]. Most members of the largest group of DNA viruses, poxviruses, encode EGF-like ligands, whose expression at sites of infection significantly increases pathogenicity[105]. RNA tumour viruses present the most divergent strategy to harness ErbB signalling: the avian erythroblastosis virus (AEV) encodes a truncated form of ErbB1 lacking most of the ectodomain and carrying many intracellular mutations. The oncoprotein v-ErbB forms ligand-independent covalent dimers at the cell surface[106]. Active mutants of various ErbB target proteins, including small GTP-binding proteins (v-Ras), adaptors (v-Crk), protein kinases (v-Src, v-Akt, v-Raf) and transcription factors (v-Jun, v-Fos), are encoded by oncogenes of different strains of retroviruses. In addition, the mouse Cas NS-1 retrovirus, which induces pre-B cell lymphomas and myeloid leukaemia, encodes a dominant active form of c-Cbl, a ubiquitin ligase that targets ErbB proteins to lysosomal degradation[102]. This interferes with receptor ubiquitylation and degradation, similar to the effect of E5, a product of the human papilloma virus (HPV) that inhibits ErbB1 degradation through inhibition of an endosomal proton-ATPase[107]. Both E5 and v-Cbl increase the rate of receptor recycling back to the cell surface.

TRABECULAE
Finger-like projections of cardiac muscle cells that form ridges in the ventricular wall.

SYMPATHETIC GANGLIA
Clusters of sympathetic neurons in which a glandular or other epithelium is embedded.

NEURAL CREST
A group of embryonic cells that separate from the embryonic neural plate and migrate, giving rise to the spinal and autonomic ganglia, peripheral glia, chromaffin cells, melanocytes and some haematopoietic cells.

CLONAL EXPANSION
Growth of a population of cells from a single precursor cell.

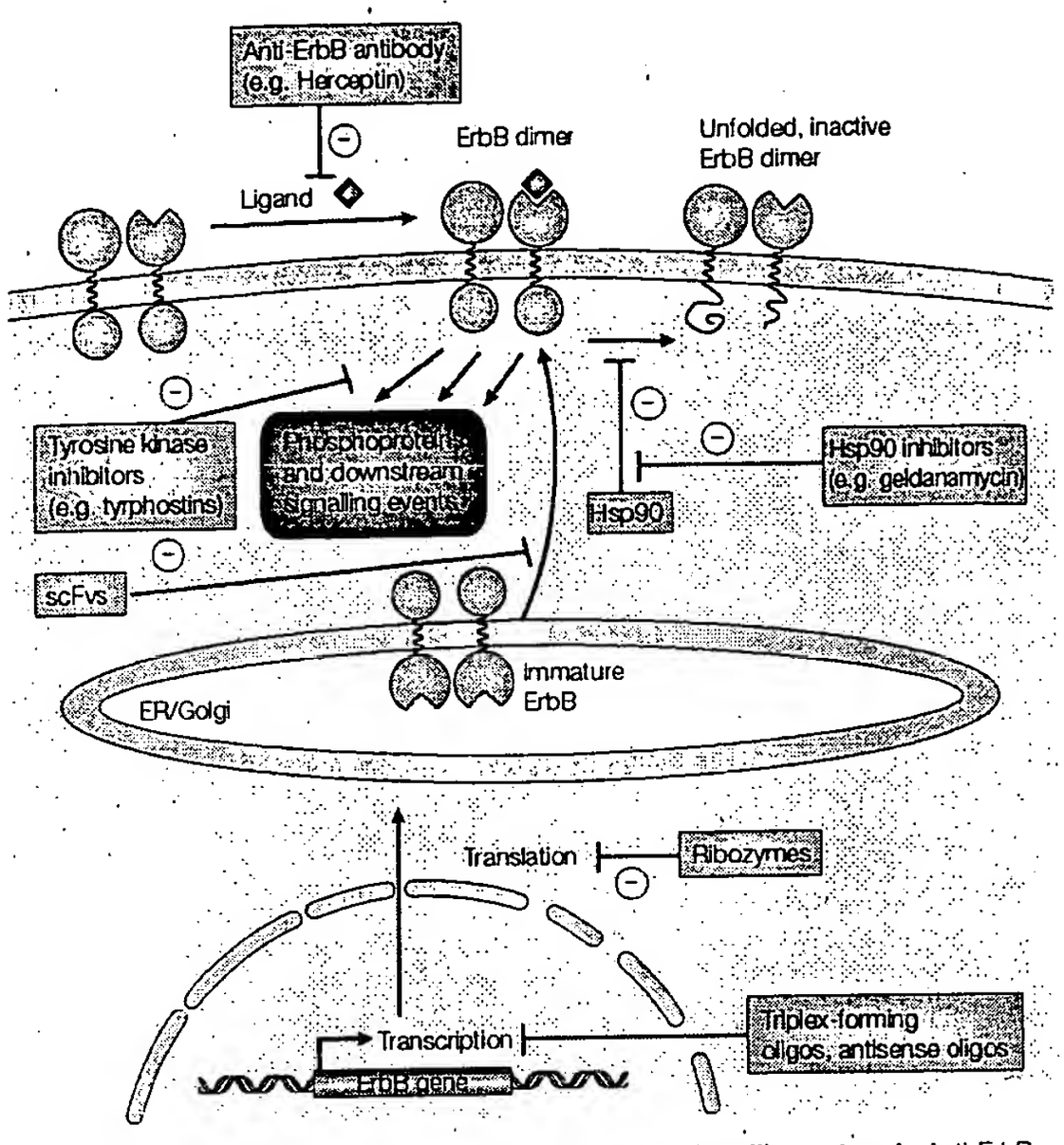


Figure 4 | **Therapeutic strategies for blocking the ErbB signalling network.** Anti-ErbB antibodies (such as Herceptin®, which binds ErbB2) block ligand binding and stimulate receptor internalization. Tyrosine kinase inhibitors such as tyrphostins block downstream signalling of the receptor–ligand complex, and Hsp90 inhibitors (for example, geldanamycin) prevent stabilization of ErbBs at the membrane. The active conformation of ErbB2 is maintained through interactions with a chaperone (Hsp90), and therefore chaperone antagonists inactivate the oncoprotein. It might also be possible to prevent ErbBs from reaching the cell surface, by blocking their transcription with triplex-forming oligonucleotides, their translation with antisense oligonucleotides or ribozymes, or their trafficking to the cell surface with intracellular single-chain Fv fragments of antibodies (scFvs). (ER, endoplasmic reticulum.)

particular tumour has an overactive ErbB pathway because of mutation, overexpression or amplification of a component of the ErbB pathway, as it can tell us what the patient's chance of survival is and with what drug they should be treated (FIG. 4).

Ligands. The relationship between ErbB ligand expression and tumorigenicity is complex: growth factors can be induced secondarily by a primary oncogene; either the stroma or the tumour can act as a ligand source; or the ligand can be expressed but unprocessed or sequestered in an inactive form[49].

Of all the ErbB ligands, the relevance of TGF-α to human cancer is best characterized. TGF-α and ErbB1 are co-expressed in several types of CARCINOMAS[50], and expression of TGF-α, particularly in lung, ovary and colon tumours co-expressing ErbB1, correlates with poor PROGNOSIS (reviewed in REF. 51). In prostate cancer, the pattern of expression of TGF-α seems to change as the disease progresses[52]. In early, ANDROGEN-DEPENDENT PROSTATE CANCER, TGF-α is expressed primarily in the tumour stroma, which suggests paracrine signalling. In advanced, androgen-independent disease, TGF-α is expressed by the tumour cells themselves, indicating AUTOCRINE signalling. Less information is available on other ligands (TABLE 1).

ErbB1. Both overexpression and structural alterations of ErbB1 are frequent in human malignancies. However, *in vitro* studies suggest that overexpression of the normal receptor leads to transformation only in the presence of a ligand. Accordingly, expression of EGF-like ligands often accompanies ErbB1 overexpression in primary tumours. Overexpression of ErbB1 is a very frequent genetic alteration in brain tumours; amplification of the gene occurs in 40% of gliomas[53]. Overexpression is associated with higher grade, higher proliferation and reduced survival. In a significant proportion of tumours, GENE AMPLIFICATION is accompanied by rearrangements. The most common mutation (type III) deletes part of the extracellular domain[35], yielding a constitutively active receptor. Recent studies identified an identical alteration in carcinomas of the lung, ovary and breast, suggesting broader implications to human cancer[54].

ErbB2. Several types of cancers overexpress ErbB2 (reviewed in REF. 56). The association of ErbB2 expression with cancer is best studied in breast cancer, where protein is overexpressed owing to gene amplification in 15–30% of invasive DUCTAL BREAST CANCERS[55]. Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, ANEUPLOIDY and lack of steroid hormone receptors, implying that ErbB2 confers a strong proliferative advantage to tumour cells[56,57]. Paradoxically, a higher degree of ErbB2 overexpression is reported in early forms of breast cancer relative to more advanced invasive carcinomas, suggesting that alterations in ErbB2 alone are insufficient for breast tumour progression from a relatively benign to a more malignant phenotype[56].

The identification of ErbB2 amplifications by FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH; FIG. 5) has now been approved by the US Food and Drug Administration to pinpoint patients at high risk for recurrence and disease-related death with node-negative invasive breast cancer[56,58]. Efforts are also being made to correlate ErbB2 status with predictive value — in other words, do patients with ErbB2 amplifications benefit from particular types of therapy? Again FISH technology can identify patients who might benefit from more aggressive therapy[59]. Several studies have shown that ErbB2 overexpression is associated with resistance to anti-oestrogen therapy[60]. Most ErbB2-overexpressing tumours do not express the oestrogen and progesterone receptors, indicating inverse relationships between the steroid hormone axis and the ErbB network.

Clinically, this crosstalk might be critical: patients treated with an anti-oestrogen drug were found to have a worse outcome if their tumours overexpressed ErbB2

CARCINOMA
A malignant tumour of epithelial origin.

PROGNOSIS
The likely outcome or course of a disease.

ANDROGEN-DEPENDENT PROSTATE CANCER
An early form of prostate cancer that is responsive to androgens and anti-androgen therapy.

AUTOCRINE
Activation of cellular receptors by ligands produced by the same cell.

GENE AMPLIFICATION
A differential increase in a specific portion of the genome. Amplification is associated with neoplastic transformation and acquisition of drug resistance.

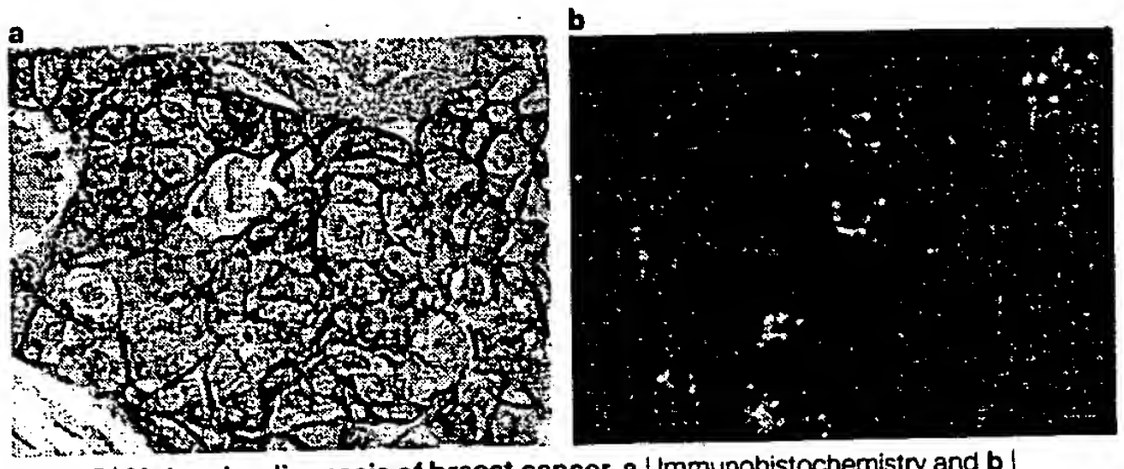


Figure 5 | **Molecular diagnosis of breast cancer. a** | Immunohistochemistry and **b** | fluorescence *in situ* hybridization (FISH) analysis of ErbB2 in human breast cancer. Immunohistochemistry was performed using HercepTest and FISH using a PathVysion ErbB2 DNA probe kit. The ErbB2 gene is seen as red fluorescence and the chromosome-17 centromeric α-satellite probe as green fluorescence. (Image courtesy of D. Eberhard, E. Huntzicker and B. Wright, Genentech, Inc.)

(REF. 61). On the one hand, *in vitro* studies indicate that overexpression of ErbB2 or NRG confers resistance to anti-oestrogens and renders cancer cells independent of oestrogen[62]. On the other hand, oestrogen suppresses transcription from the ErbB2 promoter, and specifically inhibits growth of ErbB2-overexpressing mammary cells[63]. Taken together, the molecular and clinical observations imply that the steroid and ErbB pathways are alternative, but functionally linked pathways that enhance cell proliferation (FIG. 2).

Neuregulin receptors. The catalytically inactive member of the ErbB family, ErbB3, is expressed in several cancers, but there is no evidence for gene amplification and overexpression is limited. However, a large recent study found that co-expression of ErbB2 with ErbB1 or ErbB3 in oral squamous-cell carcinoma was significant and it critically improved the predicting power[64], consistent with the non-autonomous role of ErbB3. Similarly, analysis of prostate cancer suggests the existence of a paracrine loop involving NRG1 and the ErbB2–ErbB3 heterodimer[65]. Some studies observed lower expression of ErbB4 in breast and prostate tumours relative to normal tissues, and an association with a relatively differentiated histological phenotype[66]. By contrast with epithelial tumours, childhood medulloblastomas often express ErbB4, whose co-expression with ErbB2 has a prognostic value[67], in line with the importance of receptor heterodimerization.

The network as a target for cancer therapy

The central role of the ErbB network in the development of solid tumours, its availability to extracellular manipulation, and detailed understanding of the underlying biochemistry have made the ErbB network an attractive target for pharmacological intervention (FIG. 4). Most efforts have concentrated on ErbB2 and ErbB1 owing to their increased expression in certain tumour cells relative to normal cells.

Immunological strategies. One approach — a humanized antibody to ErbB2 called Herceptin® — has been approved for clinical use, both alone and in combination with chemotherapeutic agents. In addition to downregulating surface ErbB2, Herceptin induces the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the Rb-related protein p130, which reduce the number of cells in S phase[68]. The recruitment and activation of immune effector cells to the ErbB2-overexpressing tumour might also contribute to Herceptin's mechanism of action[69].

Alternative approaches to the use of naked monoclonal antibodies to ErbBs include making antibodies toxic to cancer cells by linking them to radionuclides, toxins or prodrugs. Active immunization with portions of ErbB2 is another promising approach[70]. Monoclonal antibodies directed to a mutant form of ErbB1 (EGFRvIII) found in gliomas and carcinomas inhibit brain tumours in a manner dependent on the Fc receptor[71]. Comparison of two tumour-inhibitory monoclonal antibodies to ErbB1 revealed that only one depends on immune mechanisms; the other acts primarily by altering receptor functions. The chimeric version of this antibody, C225, competes with ligand binding to ErbB1 and arrests cultured cells at G1 because of an elevation in $p27^{KIP1}$ (REF. 72). This therapeutic antibody is now in late-stage clinical testing in patients with colorectal or head and neck cancers.

Low molecular weight inhibitors. The discovery of naturally occurring compounds capable of inhibiting the ErbB network (for example, herbimycin, genistein and emodin) led to the synthesis of analogues specific to the nucleotide-binding sites of ErbB proteins or their putative chaperones, the 90-kDa heat-shock proteins (Hsp90)[73]. The chaperone might escort ErbB proteins from the endoplasmic reticulum to the plasma membrane, where it might stabilize the active conformation of the kinase. The crystal structures of related kinases were used to enhance selectivity of synthetic tyrosine kinase inhibitors to ErbBs[73].

Both reversible and irreversible inhibitors[74] capable of discriminating between ErbBs and other kinases have been developed. When applied *in vitro* and in animal models, the compounds variably inhibited cell growth with some specificity for ErbB1- and ErbB2-expressing cells. At least five of these compounds are now being tested in human clinical studies. Because some studies indicated that Ras and Src are essential for transformation by ErbB proteins, FARNESYL TRANSFERASE INHIBITORS, Src-specific TYRPHOSTINS, MAPK inhibitors and Akt inhibitors might also be therapetically effective in containing activated ErbB pathways[75].

Gene therapy. Strategies aimed at blocking transcription, translation or maturation of ErbB transcripts or proteins are candidates for gene therapy. Early studies have shown that the adenovirus type 5 early region 1A (E1A) gene product can block ErbB2 overexpression and suppress the tumorigenic potential of ErbB2-overexpressing ovarian cancer cells[76]. This method is now being tested in a phase I trial with ovarian cancer patients. Intracellular single chain antibodies (scFvs) directed to either ErbB1 or ErbB2 can effectively inhibit

DUCTAL BREAST CANCER
Cancer arising from the lining of the milk ducts, as opposed to the lobules of the breast (lobular breast cancer).

ANEUPLOIDY
An abnormal number of chromosomes caused by their inaccurate segregation during cell division.

FLUORESCENCE *IN SITU* HYBRIDIZATION
Visualizing a genetic marker on a chromosome by using a fluorescently labelled polynucleotide probe that hybridizes to a gene on a chromosome during metaphase.

FARNESYLTRANSFERASE INHIBITORS
Inhibitors that block the activity of Ras by preventing the addition of a farnesyl group that targets it to the plasma membrane.

TYRPHOSTINS
A type of tyrosine kinase inhibitor.

receptor transfer from the endoplasmic reticulum to the plasma membrane, and thereby reduce signalling[77].

A human protocol for the treatment of ErbB2-positive ovarian cancer with scFvs has been developed following demonstration of selectivity and phenotypic effects *in vitro*[78]. Triplex-forming oligonucleotides that bind to a purine-rich sequence in the ErbB2 promoter are potent and specific inhibitors of ErbB2 transcription in an *in vitro* assay[79]. Antisense oligonucleotides[80], various dominant-negative mutants of ErbBs[81] and specific ribozymes[82] show specificity and efficacy in blocking receptor expression in cultured cells, and therefore might also prove useful as therapeutic lead compounds.

Perspectives

Successful treatments have been or are being developed to target aberrant ErbB receptor signalling in cancer; however, the potential for exploiting this pathway is still in its infancy. Antagonizing ErbB signalling might be a useful strategy for treating proliferative diseases other than cancer. One such opportunity might be coronary atherosclerosis. The migration of vascular smooth muscle cells in the arterial intima contributes to this cardiovascular disorder, particularly restenosis. Activation of the thrombin receptor is required for smooth muscle cell migration and proliferation, and activation of this G-protein-coupled receptor depends on transactivation by ErbB1 in response to heparin-binding EGF. Blockade of ErbB1 activation might therefore aid in the treatment of this disorder[83].

Another opportunity for intervention by targeted ErbB therapy might be psoriasis[84]. In normal skin, ErbB1 expression is restricted to the basal layer whereas in psoriatic skin, ErbB1 and one of its ligands, amphiregulin, are highly expressed throughout the entire epidermal layer[85]. Inhibition of ErbB1 activation might help control the spread or recurrence of psoriatic lesions.

In contrast to inhibiting ErbB signalling, potential also exists for activating the pathway in clinically meaningful ways. For example, ErbB ligands might promote wound healing[86]. ErbB signalling is also involved in fetal lung development, and appropriate activation of these pathways might benefit premature infants[87]. Neuregulins, which are also known as glial growth factors, are potent mitogens for Schwann cells[88]. Activation of Schwann cells with NRG might help resolve peripheral nerve injuries or neuropathies[89].

In summary, the ErbB field has made significant strides since Stanley Cohen's initial observation that EGF induces precocious eyelid opening in neonatal mice[90]. Although many of the individual molecules involved in ErbB signalling have been characterized, a full understanding of how the network functions in homeostasis — or malfunctions in a number of diseases — requires further definition. Regardless, the interface between basic and translational science has been established, and exploiting the ErbB pathway will probably yield other meaningful advances in the very near future.

Links

DATABASE LINKS ErbB1 | NRGs | ErbB3 | EGF | epiregulin | NRG1β | betacellulin | PI(3)K | Shc | p70S6K | PKC | Akt | Grb7 | *fos* | *jun* | *myc* | zinc finger | Sp1 | Egr1 | GABP | Grb2 | phospholipase Cγ | Src | Pyk2 | arrestin | Jak2 | interleukin-6 | TGF-α | *Drosophila* EGF receptor | NRG1 | Herceptin | $p27^{Kip1}$ | Rb | p130 | C225 | Hsp90 | amphiregulin | Vein | Gurken | Spitz | Argos

FURTHER INFORMATION The tumour gene database

ENCYCLOPEDIA OF LIFE SCIENCES *C. elegans* vulval induction | *Drosophila* embryo: dorsal–ventral specification

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Untangling the ErbB signalling network. [Review] [112 refs]
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Abstract
When epidermal growth factor and its relatives bind the ErbB family of receptors, they trigger a rich network of signalling pathways, culminating in responses ranging from cell division to death, motility to adhesion. The network is often dysregulated in cancer and lends credence to the mantra that molecular understanding yields clinical benefit: over 25,000 women with breast cancer have now been treated with trastuzumab (Herceptin), a recombinant antibody designed to block the receptor ErbB2. Likewise, small-molecule enzyme inhibitors and monoclonal antibodies to ErbB1 are in advanced phases of clinical testing. What can this pathway teach us about translating basic science into clinical use? [References: 112]

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REVIEW / SYNTHÈSE

The role of ErbB-2 tyrosine kinase recept r in cellular intrinsic chemoresistance: mechanisms and implications

Moulay A. Alaoui-Jamali, Jesse Paterson, Ala-Eddin Al Moustafa, and Lily Y n

Abstract: The erbB family of tyrosine kinase receptors is involved in the regulation of a variety of vital functions including cell proliferation, cell differentiation, and stress response. Alteration in the expression of erbB receptors occurs in numerous tumor types and plays an important role in cancer development, cancer progression, and susceptibility to cell killing by anticancer agents. Of particular interest is the intrinsic drug resistance associated with overexpression of the erbB-2 receptor. In general, tumor cells overexpressing erbB-2 are intrinsically resistant to DNA-damaging agents such as cisplatin. While the molecular mechanisms by which erbB-2 induces drug resistance are not yet established, there is evidence that this may be a consequence of altered cell cycle checkpoint and DNA repair mechanisms and dysregulation of apoptotic pathway(s). The apoptotic signal induced by many anticancer drugs originates at a receptor on the cell membrane and is transduced through a signaling cascade to the nucleus. Drug-induced apoptosis is dependent on the balance between cell cycle checkpoints and DNA repair mechanisms. Blockade of erbB-2 signaling using erbB-2 antagonists, dominant negative mutants, or chemical inhibitors of erbB-2 tyrosine kinase activity induces cell cycle arrest, inhibits DNA repair, and (or) promotes apoptosis. Less understood are downstream signal transduction cascades by which erbB-2 affects these regulatory mechanisms. The diversity of erbB receptors results in an interconnected network of cell signaling pathways that determine tumor cell fate in response to chemotherapy stress. Further investigations on the role of erbB-coupled signaling in the regulation of stress responsive genes are critical to understand the mechanisms by which tumor cells escape cell death, and will contribute to the development of alternative therapeutic targets to overcome intrins drug resistance in clinical settings.

Key words: erbB receptors, drug resistance, cell cycle checkpoints, DNA repair, apoptosis.

Résumé : La famille des récepteurs erbB à activité de tyrosine-kinase intervient dans la régulation de diverses fonctions vitales, dont la prolifération et la différenciation cellulaires, ainsi que la réponse à un stress. Une modification de l'expression de récepteurs erbB est notée dans plusieurs types de tumeurs et joue un rôle important dans le développement et la progression d'un cancer, ainsi que dans la sensibilité des cellules à des agents antitumoraux. La résistance intrinsèque aux médicaments est particulièrement associée à une surexpression du récepteur erbB-2. Généralement, les cellules tumorales surexprimant erbB-2 sont résistantes à divers médicaments antitumoraux, dont le cisplatine. Alors que nous ne connaissons pas encore les mécanismes moléculaires par lesquels erbB-2 induit une résistance aux médicaments, des éléments indiquent que cela pourrait être la conséquence d'une modification d'un contrôle du cycle cellulaire et des mécanismes de réparation de l'ADN et d'une mauvaise régulation d'une des voies d'apoptose. Le signal d'apoptose induit par plusieurs médicaments antitumoraux est issu d'un récepteur à la surface des cellules et est transduit jusqu'au noyau par une cascade de signaux. L'apoptose induite par un médicament est fonction de l'équilibre entre les contrôles du cycle cellulaire et les mécanismes de réparation de l'ADN. Le blocage du signal issu d'erbB-2 par des antagonistes d'erbB-2, des mutants négatifs dominants ou des inhibiteurs de l'activité tyrosine-kinase induit un arrêt du cycle cellulaire, inhibe la réparation de l'ADN ou entraîne

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Abbreviations: EGFR, epidermal growth factor; MAP, mitogen-activated protein; PKC, protein kinase C; SAPK, stress-activated ein kinase.

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l'apoptose. Les cascades de transduction du signal par lesquelles erbB-2 affecte ces mécanismes de régulation sont moins bien connues. La diversité des récepteurs erbB engendre un réseau de voies de signalisation cellulaire reliées entre elles, qui détermine le sort des cellules tumorales à la suite d'une chimiothérapie. Une étude plus poussée du rôle des signaux issus d'erbB dans la régulation des gènes sensibles à un stress est essentielle à la compréhension des mécanismes par lesquels les cellules tumorales échappent à la mort cellulaire et va contribuer à définir de nouvelles cibles thérapeutiques afin de déjouer la résistance intrinsèque aux médicaments.

Mots clés : récepteurs erbB, résistance aux médicaments, contrôles du cycle cellulaire, réparation de l'ADN, apoptose.

[Traduit par la rédaction]

Intr duction

The erbB-2 receptor belongs to the type 1 tyrosine kinase receptor family, which also includes epidermal growth factor (EGFR), erbB-3, and erbB-4. These receptors have attracted a great deal of interest because of their biological diversity, their frequent alteration in cancer, and their possible utility as prognostic factors (Ludes-Meyers et al. 1996; Tanner et al. 1996; Smit et al. 1996; Onda et al. 1996; Brandt et al. 1995). Overexpression of the wild-type erb-B2 induces malignant transformation of human bronchial and mammary cells as well as rodent fibroblast cells (Noguchi et al. 1993; Zhai et al. 1993; Pierce et al. 1991; Di Fiore et al. 1987). Activation of the neu gene (the rat homologue of human erbB-2) by a single substitution of valine at position 664 to glutamic acid led to oncogenic transformation and development of mammary tumors (reviewed in Hynes and Stern 1994; Bargmann et al. 1986). Transgenic mice expressing an inducible wild-type erbB-2 developed cancer such as lung adenocarcinomas and lymphomas (Suda et al. 1990). In humans, the overexpression–amplification of erbB receptors has been found in a wide variety of tumor types including lung, ovarian, breast, and kidney carcinomas (Table 1). In many cases, the overexpression of erbB receptors was reported to predict a poor prognosis and shorter survival. In vitro studies also indicate that cells derived from these tumors are intrinsically resistant to drugs, compared with cells from tumors with low erbB-2 expression. The main purpose of this article is to provide an overview of the current knowledge and therapeutic implications of the mechanisms by which overexpression of the erbB-2 tyrosine kinase receptor leads to intrinsic drug resistance.

Bi l gy of the erbB family of tyrosine kinase receptors

The erbB receptors are characterized by the presence of an extracellular ligand binding domain, extracellular cysteine-rich domains, one transmembrane domain, an intracellular tyrosine kinase domain, and autophosphorylation sites. Four members of the erbB receptor family have been identified and include the epidermal growth factor (EGFR or erbB-1; $p170^{EGFR}$), erbB-2 ($p185^{HER2/erbB2}$), erbB-3 ($p160^{HER3/erbB3}$), and erbB-4 ($p180^{HER4/erbB4}$). Upon ligand binding, the erbB receptors homodimerize or heterodimerize, resulting in receptor phosphorylation and transphosphorylation and subsequent activation of multiple signaling cascades. The formation and activation of heterodimers constitute an important regulatory process that occurs in a hierarchical network of interreceptor interactions (Fig. 1) (Tzahar et al. 1996; Riese et al. 1995).

Unlike the other erbB receptors, no specific ligand has yet been found for the orphan erbB-2 receptor. However, EGFR ligands such as EGF and TGFα can act as agonists to stimulate tyrosine phosphorylation of erbB-2 through heterodimerization (Fig. 1) (Pinkas-Kramarski et al.1996*a*; Wada et al. 1990; Goldman et al. 1990; reviewed in Hynes and Stern 1994). The ligands for erbB-3 and erbB-4 are collectively termed heregulins (also referred to as neuregulins or neu differentiation factors). Heregulins were found to exist as various isoforms resulting from alternative splicing of a single gene mapped to human chromosome 8p22-p11, with isoform expression following a tissue-specific pattern (Chang et al. 1997; Carraway et al. 1997; Lee and Wood 1993; Holmes et al. 1992). The most characterized heregulin is a 44-kDa glycoprotein that exists in multiple isoforms subdivided into two groups, α and β, on the basis of their EGFR-like domains. Heregulins bind to erbB-3 and erbB-4 with low and high affinity, respectively (Earp 1995), and also interact with heterodimers between EGFR/erbB-3, EGFR/erbB-4, erbB-2/erbB-3, and erbB-2/erbB-4 (Tzahar et al. 1996; Pinkas-Kramarski et al. 1996*a*, 1996*b*; Bacus and Yarden 1996; Carraway and Cantley 1994; Peles et al. 1993). Heregulins induce phosphorylation of erbB-2 through transphosphorylation and (or) receptor heterodimerization (Fig. 1) (Karunagaran et al. 1996, 1995; Riese et al. 1995; Carraway et al. 1994, Carraway and Cantly 1994; Plowman et al. 1993). Furthermore, erbB-2 is the preferred heterodimer partner of the three other members of the erbB family, and it is essential for signal transmission by heregulins through the kinase defective erbB-3, which is devoid of any biochemical activity when expressed alone (Chen et al. 1996; Guy et al. 1994).

Co-expression of erbB-2/erbB-3 or erbB-2/EGFR heterodimers was reported to cooperate in the transformation of NIH-3T3 cells (Kokai et al. 1989; Kraus et al. 1993; Alimandi et al. 1995). Also, it has been shown that the erbB-2/erbB-3 heterodimer is the major receptor complex that binds heregulins on mammary epithelial cells, whereas the EGFR/erbB-2 heterodimer or the EGFR homodimer predominantly binds EGF (Chen et al. 1996). Furthermore, erbB-2 was reported to enhance the binding affinities of heregulins and EGF to their receptors by diminishing the rates of ligand dissociation (Karunagaran et al. 1996). Consistently, the relatively low ligand binding affinity of erbB-3 is augmented by co-expression of erbB-2 (Sliwkowski et al. 1994), and the abolishment of erbB-2 expression at the cell surface severely impairs ligand binding and erbB signaling (Karunagaran et al. 1996; Graus-Porta et al. 1995). These observations suggest that EGFR, erbB-3, and erbB-4 receptors compete for interaction with erbB-2.

Table 1. Incidence of erbB-2 overexpression in human cancers.

Tumor type	Incidence (%)	Source
Non-small cell lung cancer	25–55	Kern et al. 1990; Schneider et al. 1989; Shi et al. 1992
Breast	15–35	Slamon et al. 1989; Iglehart et al. 1990; Borg et al. 1990; Bacus et al. 1990
Ovarian	30	Berchuck et al. 1990; Slamon et al. 1989
Esophagus	60–70	Al-Kasspooles et al. 1993; Jankowski et al. 1992
Gastric	5–55	Kameda et al. 1990; Yokota et al. 1986
Colon	7	D'Emilia et al. 1989
Bladder	35	Sauter et al. 1993
Kidney	40	Yokota et al. 1986; Weidner et al. 1990
Prostate	30*	Arai et al. 1997
Osteosarcoma	40	Onda et al. 1996
Salivary	30–60	Stenman et al. 1991

* The percentage of patients with high serum erbB-2 in advanced stage D2.

erbB-coupled cell signaling

Many critical components that link extracellular signals to the nucleus are coupled to the erbB tyrosine kinase receptors (Fig. 1). These include the Ras/Raf/ERK kinase pathway, phospholipase C-gamma (PLC-γ) (Carpenter et al. 1992; Hernandez-Sotomayor and Carpenter 1992), phosphatidylinositol-3-kinase (PI-3-K), Src, p70 S6 kinase, and STAT (Carpenter and Cantley 1996; Rodriguez-Viciana et al. 1996; Kapeller and Cantley 1994; also reviewed in Dougall et al. 1994). Activation of erbB-2 leads to a strong stimulation of the Ras/Raf/ERK protein kinase cascade and the induction of c-fos and c-jun (Sepp-Lorenzino et al. 1996; Ben-Levy et al. 1994; Satoh et al. 1990; Sistonen et al. 1989). Stimulation of erbB-2 by heregulins or EGF also stimulates the activity of the stress-activated protein kinase (SAPK) subfamily of MAP kinases (reviewed in Dougall et al. 1994). The activation of rat neu by a point mutation in the transmembrane domain causes constitutive tyrosine kinase activity and induces transcriptional activation of a variety of transcription factors including AP1, Ets, and NF-κB-dependent genes. This activation seems to occur primarily via the Ras/Raf signaling pathway because Ras or Raf dominant negative mutants blocked the neu-mediated transcriptional activation (Galang et al. 1996).

Unlike erbB-2, the erbB-3 and erbB-4 receptors are more potent in activating PI-3-K than the Ras/Raf/ERK pathway (Ram and Ethier 1996; Sepp-Lorenzino et al. 1996). This may be attributed to the presence of multiple putative binding sites for PI-3-K binding on erbB-3 and erbB-4 cytoplasmic domains (Carraway and Cantly 1994).

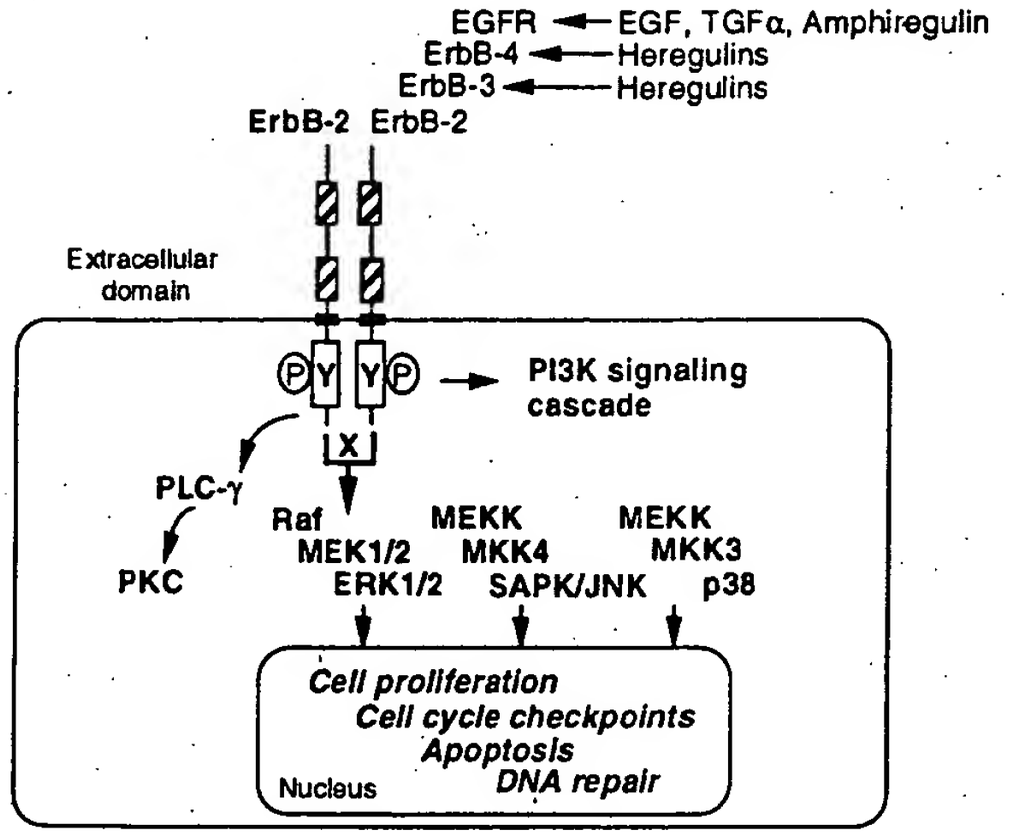
Protein kinases also play a role in the regulation of erbB receptor activities. Activation of protein kinase C (PKC) leads to phosphorylation of erbB-2 and EGFR cytoplasmic domains at serine–threonine residues, decreasing the ligand binding affinity and tyrosine kinase activity of these receptors (Cao et al. 1991; Magun et al. 1980). PKC stimulates the cleavage of the transmembrane forms of TGFα (Pandiella and Massague 1991) and heparin-binding EGF (Raab et al. 1994) to produce diffusible, extracellular, and low molecular weight ligands. This inhibits EGFR activity. PKC activators such as phorbol esters (e.g., 12-*O*-tetradecanoylphorbol-13-acetate) also act at the level of the ligand by stimulating transcription of mRNA encoding EGFR ligands (Dluz et al. 1993; Shoyab et al. 1988). Furthermore, downregulation of the erbB-4 receptor, but not EGFR, erbB-2, or erbB-3, by PKC activators has been reported to occur as a result of a proteolytic cleavage of erbB-4, producing an 80-kDa cytoplasmic domain with low levels of phosphotyrosine, and a 120-kDa ectodomain fragment that is released into the extracellular medium. The 80-kDa cytoplasmic domain fragment that is membrane associated is recognized by antibody to the erbB-4 carboxyl terminus but has no tyrosine kinase activity (Vecchi et al. 1996).

Role of erbB-2 overexpression in drug resistance

Among members of the erbB family, overexpression of erbB-2 is very common in human cancers (Table 1) and has been associated with a poor prognosis and shorter survival in the clinical setting (Smit et al. 1996; Saffari et al. 1995; Shi et al. 1992; Weidner et al. 1990; Kern et al. 1990; Berchuck et al. 1990; Kameda et al. 1990; Iglehart et al. 1990; Slamon et al. 1989; Schneider et al. 1989; Yokota et al. 1986). It has been proposed that the association of erbB-2 with high tumor proliferation and poor prognosis may be the result of (*i*) the unique high basal tyrosine kinase activity of erbB-2 compared with other erbB receptors, (*ii*) the superior activity of erbB-2 to form heterodimers with the other erbB receptors, (*iii*) the multiplicity of potential docking sites for SH2 proteins in the erbB-2/erbB-3 heterodimer compared with other heterodimers, and (*iv*) a prolonged receptor phosphorylation as a result of possible saturation of phosphatases, required for phosphotyrosine turnover, by the erbB-2 receptor (Tzahar et al. 1996; reviewed by Hynes and Stern 1994).

Studies using cell lines derived from lung cancer have shown that the level of expression of erbB-2 is positively correlated with enhanced cellular resistance to a variety of anticancer drugs including cisplatin (Figs. 2 and 3) (Paterson et al. 1996; Zhang and Hung 1996; Tsai et al. 1993; unpublished data from this laboratory). Similar results have been reported on breast and ovarian adenocarcinoma cells (Arteaga et al. 1994; Pietras et al. 1994; Benz et al. 1993). Resistance to cisplatin may involve various mechanisms including the alteration of drug transport, drug metabolism, cell cycle checkpoint and DNA repair mechanisms, and (or) an imbalance between apoptotic versus anti-apoptotic proteins. Neither intracellular drug accumulation nor the expression of detoxification enzymes, e.g., glutathione and glutathione *S*-transferases, were affected by the overexpression of erbB-2 (unpublished data from this laboratory; Paterson et al. 1996; Arteaga et al. 1994;

Fig. 1. ErbB-2 homodimer and heterodimer formation. ErbB-2 is an orphan receptor that can form erbB-2/erbB-2 homodimers. Ligand binding to EGFR, erbB-3, and erbB-4 induces the formation of erbB-2/EGFR, erbB-2/erbB-3, or erbB-2/erbB-4 heterodimers. Activation of erbB-2 results in the autophosphorylation of its cytoplasmic tail. The phosphotyrosines can now serve as the docking site for many SH2-containing proteins such as the adapter protein Grb2. Grb2's double SH3 domains are constitutively associated with son of sevenless (SOS) protein and the recruitment of Grb2 to the membrane is believed to localize SOS to the membrane. SOS exchanges GDP for GTP on $p21^{ras}$. GTP-Ras recruits the serine protein kinase Raf to the plasma membrane, where it initiates a sequential activation of several cytoplasmic kinases, referred to as the mitogen-activated protein kinases (MAPKs), also described as extracellular signal-regulated kinases (ERKs). Other kinases that can be activated through pathways dependent on Ras but not Raf include JNK/SAPK and p38. PLC-γ and PI-3-kinase as well as STAT91/ISGF-3 are also coupled to erbB-2 (reviewed in Hynes and Stern 1994; Dougall et al. 1994).



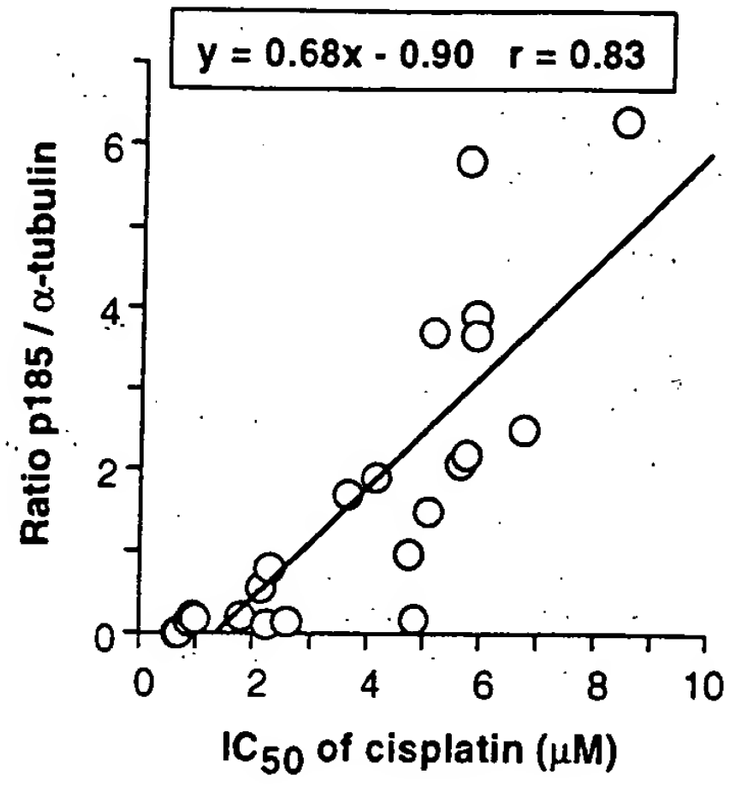
Pietras et al. 1994). However, dysregulation of cell cycle checkpoint, DNA repair, and (or) apoptosis mechanisms seem to play an important role.

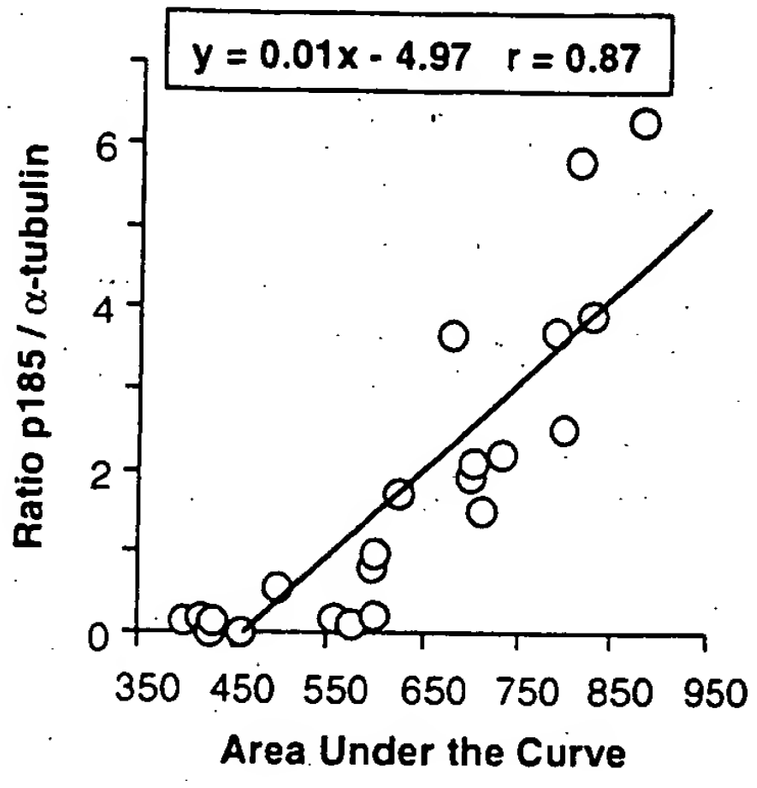
Overexpression of erbB-2 is associated with high mitogenic activity (Bacus et al. 1990; Di Fiore et al. 1987). The erbB-coupled signaling pathways are involved in gene regulation of cell cycle checkpoint controls (Dougall et al. 1994; Christen et al. 1994; Bhushan et al. 1992; Kashani-Sabet et al. 1990). Cell cycle checkpoints have been shown to be induced as a common cellular response to DNA damage induced by drugs and are believed to be critical for both DNA repair and apoptosis (Hartwell and Kastan 1994). Treatment of cells overexpressing erbB-2 with heregulins as well as antibodies against erbB-2 increases the intracellular level of p53 (Bacus et al 1996). The associated cell cycle delay seems to occur via the induction of the cyclin-dependent kinase inhibitor p21 (WAF1/Cip1). $p21^{WAF1}$ is induced in response to DNA damage in a p53-dependent manner (El-Deiry et al. 1994), although a p53-independent induction of $p21^{WAF1}$ has also been reported (Gartenhaus et al. 1996; Kondo et al. 1996; Marchetti et al. 1996; Russo et al. 1995; Macleod et al. 1995). DNA repair defects were observed in $p21^{WAF1-/-}$, compared with $p21^{WAF1+/+}$ cells, as a result of altered checkpoint mechanisms because $p21^{WAF1-/-}$ cells were defective in the G_1 checkpoint following DNA damage (Deng et al. 1995; Serrano et al. 1995; Li et al. 1994). Also, the replacement of $p21^{WAF1}$ rescued DNA repair and this appears to be dependent on the presence of the PCNA-interacting C-terminal domain of $p21^{WAF1}$ (MacDonald et al. 1996; Li et al. 1996). These observations support earlier work describing the requirement of cell cycle checkpoints for DNA repair.

Evidence for direct involvement of DNA repair in erbB-2-induced drug resistance comes from this laboratory (Yen et al. 1997; Paterson et al. 1996) as well as others (Arteaga et al. 1994; Pietras et al. 1994). Cancer cells that constitutively overexpressed erbB-2 or were engineered to overexpress the human erbB-2 were found to be resistant to anticancer drugs such as cisplatin, adriamycin, and VP16 (Figs. 2 and 3). They also had an enhanced overall DNA repair capacity (Fig. 2) (Yen et al. 1997; Zeng-Rong et al. 1995; unpublished data from this laboratory), predictably involving nucleotide excision repair, a pathway by which cells remove bulky type adducts such as those induced by cisplatin (Sancar 1996; Huang et al. 1994). This has also been shown in breast, ovarian, and cervical cancer cells overexpressing erbB-2 or EGFR receptors (Arteaga et al. 1994; Pietras et al. 1994; Benz et al. 1993; Johnson et al. 1993; Nishikawa et al. 1992; Zhen et al. 1992). Another study reported that activation of the erbB member EGFR by EGF enhances the cytotoxic effect of cisplatin and UV light by a mechanism involving downregulation of DNA repair (Christen et al. 1994). Furthermore, gemcitabine, a nucleoside analogue that interferes with DNA repair synthesis, was found to synergize efficiently with cisplatin in cells overexpressing erbB-2, as compared with cells with low erbB-2 expression (Tsai et al. 1996; Shepherd et al. 1996; Steward et al. 1996; Peters et al. 1996). Although these observations support a connection between erbB-2 and DNA repair, it is still unknown which erbB signaling cascade initiates these regulatory mechanisms and which protein(s) of DNA repair pathway is (are) involved. We have shown that the expression of a ras dominant negative mutant, Asn-17-Ras^H, prevents the DNA repair inhibition induced by the anti-erbB-2 antibody in NIH-3T3 cells overexpressing erbB-2 (Yen et al. 1997). A previous study reported that phosphorylation–dephosphorylation at serine–threonine may be required for the regulation of NER proteins (Ariza et al. 1996). It is likely that the erbB-coupled signaling plays an important role in the regulation of DNA repair, as many transcription factors regulated by ras signaling, for example (reviewed in Marshall 1995), are found in consensus sequences of DNA repair genes (e.g., AP sites, Sp1, SRE).

The modulation of apoptosis by erbB-2 signaling is supported by the fact that (*i*) anti-erbB-2 antibodies induce apoptosis through a p53-dependent pathway in breast cancer cells

Fig. 2. Drug resistance and DNA repair capacity in a panel of non-small cell lung cancer cell lines expressing various levels of erbB-2. The ratio of expression of erbB-2 to α-tubulin was determined in various cell lines using Western blot analysis. The cytotoxic effect of cisplatin in these cell lines was determined following continuous exposure to cisplatin for 96 h, and cytotoxicity was determined using the MTT assay (Zeng-Rong et al. 1995). The concentration of drug that inhibited 50% of cell growth (IC_{50}) was determined from the dose-response curve for each cell line. DNA repair capacity was estimated using the host cell reactivation assay. Cells were cotransfected with pRSV-CAT plasmid pretreated with cisplatin and untreated pRSV-β-gal plasmid (control). Forty-eight to 72 h after transfection, cells were collected and the chloramphenicol acetyl transferase (CAT) activity was determined as described (Zeng-Rong et al. 1995; Yen et al. 1997). Area under the curve was calculated from the CAT activity curve generated for each cell line transfected with plasmid bearing various amounts of DNA damage. Each point represents the average of three independent determinations. The indicated graphs show positive correlation between resistance to cisplatin, the level of erbB-2 (p185), and cell capacity to reactivate CAT expression from the reporter gene (reported in part in Paterson et al. 1996).



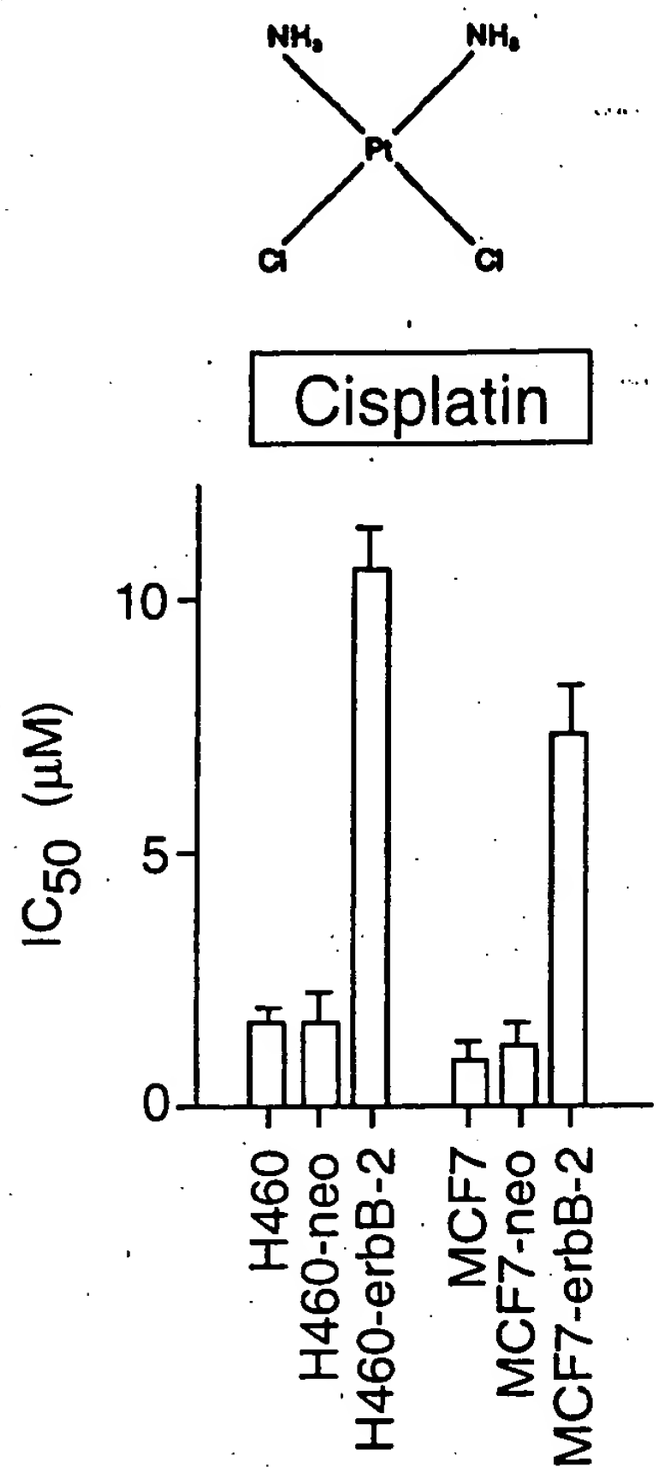


(Bacus et al. 1996), (*ii*) anti-erbB-2 intracellular single-chain antibodies also induce apoptosis in ovarian cancer, secondary to the ectopic localization of the erbB-2 protein (Deshane et al. 1996), and (*iii*) overexpression of erbB-2 in estrogen receptor proficient cells results in cell resistance to endocrine therapy and upregulation of the anti-apoptotic genes Bcl2 and Bcl-XL (Kumar et al. 1996). Drugs such as cisplatin, adriamycin, alkylating agents, as well as many biological agents activate erbB receptors and modulate various signaling pathways including the mitogen-activated protein (MAP) kinases ERK1/ERK2, and the SAPKs (Das and White 1997; Zanke et al. 1996). SAPK was implicated in growth arrest and apoptosis (Xia et al. 1995). Blockade of SAPK was shown to confer resistance to apoptosis induced by cisplatin (Zanke et al. 1996). The erbB ligands, heregulins, were also shown to regulate cell death in Schwann cells, the myelin-forming glial cells (Syroid et al. 1996). These connections between erbB signaling and apoptosis are not unanimous, as other studies have shown an inverse correlation between erbB-2 and expression of anti-apoptotic genes such as Bcl2 (Leek et al. 1994; Gee et al. 1994). The tumor growth inhibitory anti-erbB-2 antibodies primarily exert cytostatic rather than cytotoxic effects, which led to their use in combination with chemotherapy, suggesting that erbB-2 antibody-induced apoptosis may not be a general mechanism (Rusch et al. 1996) and therefore modulation of drug response by anti-erbB-2 may depend on other mechanisms such as DNA repair.

The intricate relationships between cell cycle, DNA repair, and apoptosis provide evidence that cells are able to intimately coordinate these mechanisms in response to DNA damage (Fig. 4) (Kroemer et al. 1995; Hoeijmakers et al. 1996). This interconnection may explain erbB-2-induced cross-resistance to drugs other than cisplatin and that may not trigger primarily DNA repair; for example, taxol induces primarily microtubule stabilization, and VP16 and adriamycin interfere with the topoisomerase II enzyme. Indeed, it has been reported that microtubule stabilization may not be the only mechanism by which taxol exerts its effect because the tyrosine kinase inhibitors genestein and herbimycin A prevented apoptosis induced by this drug (Liu et al. 1994). The main target of VP16 and adriamycin is the topoisomerase II enzyme, which plays a role in repair of chromosomal DNA (Thielmann et al. 1993), but has also been found to be coamplified with erbB-2 in breast cancer tissues (Smith et al. 1993).

Proteins that may play a pivotal role in the control of the cell cycle, DNA repair, and apoptosis include XPB (also referred to as ERCC3), XPD (also referred to as ERCC2), and p53 proteins. XPD and XPB are two nucleotide excision repair proteins that serve as subunits of the basal transcription factor TFIIH/BTF2 (Weeda et al. 1997; Schaeffer et al. 1993, 1994; reviewed in Hoeijmakers et al. 1996). TFIIH is implicated in cell cycle control (Hoeijmakers et al. 1996; Nigg 1995). The typical rise in p53 level following exposure to erbB antagonists or DNA damage may mediate (*i*) G1-S checkpoint arrest through negative interaction with RPA and (or) inhibition of cyclin-dependent kinases, (*ii*) stimulation of DNA repair by inducing the Gadd 45 gene, or (*iii*) triggering of apoptosis (Polyak et al. 1996; reviewed in Bates and Vousden 1996). p53 associates with XPB and XPD (Leveillard et al. 1996; Wang et al. 1995, 1996). The C-terminus of p53 binds directly to the N-terminal half of the XPD helicase, and the related domain of XPB. The C-terminus of p53 protein is involved in

Fig. 3. Drug resistance induced by stable expression of erbB-2 in tumor cells with low levels of erbB-2. The non-small cell lung cancer cell line H460 and breast cancer cell line MCF7 express very low levels of erbB-2. Transfection of these cells by the human erbB-2 gene conferred drug resistance (some of these cell lines were provided by collaborators, see Acknowledgments). Cytotoxicity was determined as described in Fig. 2.

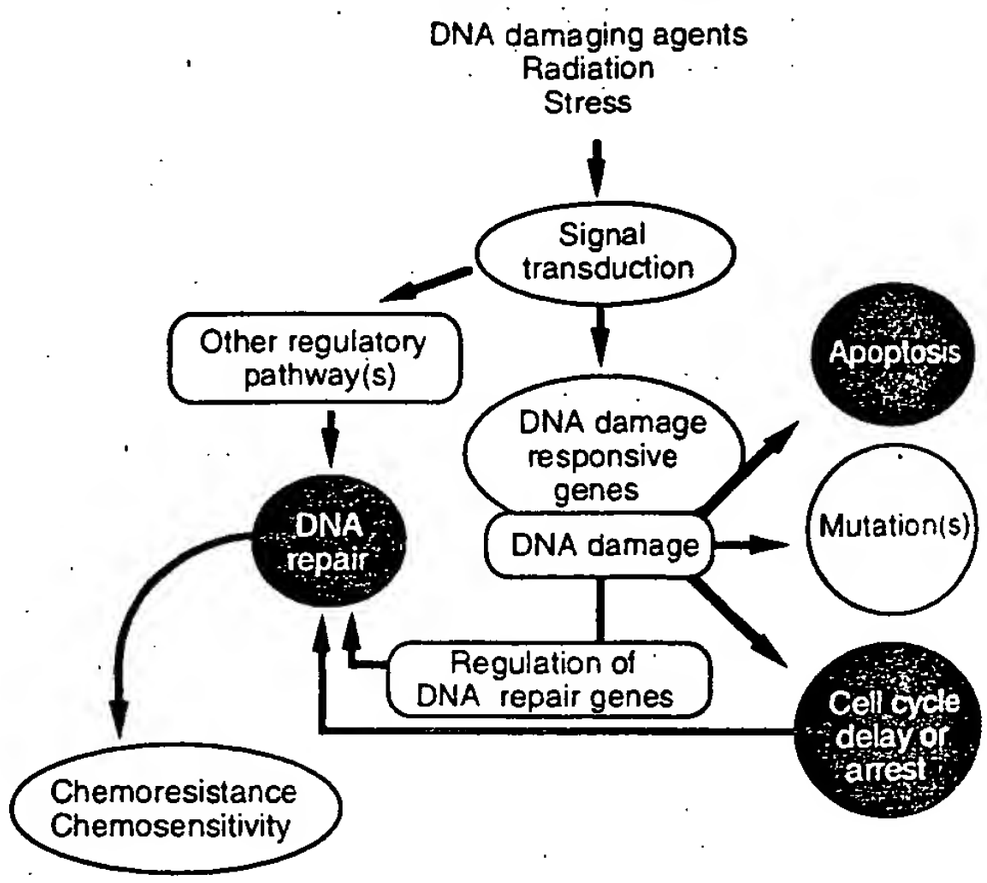


oligomerization, regulation of specific binding, single-strand re-annealing, nuclear localization, and binding to a variety of transcription factors. The N-terminus of XPB–XPD contains the ATP binding site that is required for DNA repair. Additional support of these interrelationships is provided by other observations showing that (*i*) p53-deficient mice were more susceptible to UV-induced skin cancer and have downregulation of DNA repair without alteration of UV-induced apoptosis (Tli et al. 1996), and (*ii*) inactivation of p53 by the human papillomavirus E6 oncoprotein or a dominant-negative mutant p53 transgene resulted in inhibition of in vitro DNA repair (Smith et al. 1995). These observations suggest that cell cycle checkpoints and induction of apoptosis may represent independent mechanisms (Polyak et al. 1996; reviewed in Vitetta and Uhr 1994), or more likely that cell cycle, DNA repair, and apoptosis are tightly regulated through balances that determine the fate of tumor cells to trigger or overcome cell death (Fig. 4).

Conclusion and future directions

Intrinsic resistance associated with erbB-2 overexpression seems

Fig. 4. Interrelationship between growth factor modulation, DNA repair, apoptosis, and drug resistance. Treatment with anticancer drugs stimulates specific growth factor receptors, resulting in the activation of signaling cascade(s). This (these) cascade(s) will lead to the regulation genes responsive to stress and DNA damage. Cells will either trigger apoptosis or repair DNA damage. The latter is dependent on cell cycle checkpoints that allow time for repair. If the apoptotic signal predominates, the cells will succumb to the cytotoxic effects of drugs and die (chemosensitivity). On the other hand, if DNA repair predominates, the cells will remove DNA damage, overcome apoptosis, and survive (chemoresistance).



to be multifactorial, involving cell cycle, DNA repair, and apoptosis mechanisms. Future investigations aimed at identifying regulatory mechanisms by which erbB tyrosine kinase receptors affect genes involved in these processes are critical to understand intrinsic drug resistance in the more complex heterogenous solid tumors. The ultimate goal in cancer pharmacology is to selectively trigger apoptosis in tumor cells. Drugs that target apoptosis are broadly defined as those that interfere with cell cycle, with anti-apoptotic genes and proteins, or with biochemical pathways that regulate apoptosis. In the pharmacological context, selective inhibition of DNA repair mechanisms should also promote the accumulation of DNA damage and induction of cell death. Very few compounds are known to interfere selectively with DNA repair. Inhibitors of DNA repair synthesis, e.g., aphidicolin analogues, have limitations because this step involves ubiquitous and abundant proteins that are also essential for cell replication. Current efforts to characterize DNA repair proteins and their regulatory mechanisms will open up an opportunity to design selective inhibitors. Signaling modulators may be useful alternatives to target specific erbB-2-signaling components involved in the regulation of DNA repair and apoptosis. For example, tyrosine kinase inhibitors such as herbimycin analogues (Miller et al. 1994; Yen et al. 1997), geldanamycin (Stebbins et al. 1997; Chavany et al. 1996), or emoldin (Zhang and Hung 1996) have been shown to promote degradation of erbB-2, to induce an antiproliferative activity, and (or) to enhance cellular response to chemotherapy. The diversity of erbB-2 receptor heterodimerization with the other members of the family also

offers obvious opportunities to develop inhibitors that disrupt or prevent specific protein–protein interactions. The selective overexpression and extracellular localization of the erbB receptors in cancer also offers various selective targeting strategies to deliver these modulators, such as the design of erbB-2 antibody–drug conjugates, liposome–drug–antibody conjugates, and peptide-based synthetic molecules. Many of these approaches are already under clinical trials.

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Abstract
The erbB family of tyrosine kinase receptors is involved in the regulation of a variety of vital functions including cell proliferation, cell differentiation, and stress response. Alteration in the expression of erbB receptors occurs in numerous tumor types and plays an important role in cancer development, cancer progression, and susceptibility to cell killing by anticancer agents. Of particular interest is the intrinsic drug resistance associated with overexpression of the erbB-2 receptor. In general, tumor cells overexpressing erbB-2 are intrinsically resistant to DNA-damaging agents such as cisplatin. While the molecular mechanisms by which erbB-2 induces drug resistance are not yet established, there is evidence that this may be a consequence of altered cell cycle checkpoint and DNA repair mechanisms and dysregulation of apoptotic pathway(s). The apoptotic **signal** induced by many anticancer drugs originates at a receptor on the cell membrane and is transduced through a **signal**ing cascade to the nucleus. Drug-induced apoptosis is dependent on the balance between cell cycle checkpoints and DNA repair mechanisms. Blockade of erbB-2 **signal**ing using erbB-2 antagonists, dominant negative mutants, or chemical inhibitors of erbB-2 tyrosine kinase activity induces cell cycle arrest, inhibits DNA repair, and (or) promotes apoptosis. Less understood are downstream **signal transduction** cascades by which erbB-2 affects these regulatory mechanisms. The diversity of erbB receptors results in an interconnected network of cell **signal**ing pathways that determine tumor cell fate in response to chemotherapy stress. Further investigations on the role of erbB-coupled **signal**ing in the regulation of stress responsive genes are critical to understand the mechanisms by which tumor cells escape cell death, and will contribute to the development of alternative therapeutic targets to overcome intrinsic drug resistance in clinical settings. [References: 138]

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REVIEW

Molecular signals in anti-apoptotic survival pathways

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Drug resistance, to date, has primarily been attributed to increased drug export or detoxification mechanisms. Despite correlations between drug export and drug resistance, it is increasingly apparent that such mechanisms cannot fully account for chemoresistance in neoplasia. It is now widely accepted that chemotherapeutic drugs kill tumour cells by inducing apoptosis, a genetically regulated cell death programme. Evidence is emerging that the exploitation of survival pathways, which may have contributed to disease development in the first instance, may also be important in the development of the chemoresistance. This review discusses the components of and associations between multiple signalling cascades and their possible contribution to the development of neoplasia and the chemoresistant phenotype. *Leukemia* (2001) 15, 21–34.
Keywords: apoptosis; survival pathways; chemoresistance; neoplasia

Introduction

Resistance to chemotherapy is a major concern, and a serious limiting factor in the treatment of many neoplasias including leukaemias. Initially responsive to treatment, many disorders become increasingly more drug resistant following primary or subsequent relapses, until inevitably, the elevated concentrations of cytotoxins required to control the disease can no longer be tolerated by the patient. In some situations, the treatment may be perceived as more debilitating than the disease itself. Novel pharmaceutical agents which provide a more specific treatment regimen or increase the efficacy of conventional chemotherapy, without increasing toxicity towards patients, would clearly be of immense clinical benefit.

It is now widely accepted that chemotherapeutic drugs kill tumour cells primarily through the induction of apoptosis. Apoptosis is an internally directed, energy requiring suicide programme, normally activated in response to cellular damage, or following physiological insults such as death receptor ligation[1] or withdrawal of survival signals.[2] In chemosensitive cells, drug-mediated injury is frequently translated into an instruction to initiate the apoptotic cascade. Drug resistance can, therefore, be described as a consequence of failure of tumour cells to engage apoptosis in the presence of cytotoxic drugs. Indeed, many tumours intrinsically resistant to chemotherapy are defective in their ability to activate the apoptotic machinery. Multiple inter-connecting signalling pathways are known to regulate apoptosis, survival and proliferation, including Ras, PI3-kinase/PKB, PKC, the stress-activated protein kinases, the Bcl-2 family and Fas/CD95. Each of these pathways will be discussed in this review, as defects in these pathways can have potentially harmful repercussions over tissue homeostasis, promoting oncogenesis and multidrug resistance.

Previously described mechanisms of drug resistance include expression of members of the ATP-binding cassette (ABC) superfamily of transporter proteins, multidrug resistance-associated protein (MRP), and P-glycoprotein.[3–5] This family reduces intracellular drug accumulation to sublethal levels, by active drug extrusion from the cell against a concentration gradient. Glutathione S-transferases limit drug-mediated damage by conjoining drugs with reduced glutathione prior to exportation from the cell.[3,6] The target of many cytotoxins is DNA, therefore increased activity of DNA repair enzymes to minimise inflicted damage, is a further established mechanism of drug resistance. However, despite several studies reporting an association between drug export and drug resistance, it is apparent that such mechanisms cannot be solely responsible for chemoresistance and treatment failure.[7] Drug resistance may also arise following multiple courses of chemotherapy where increasingly drug-resistant or anti-apoptotic clones are positively selected.

Attempts to elucidate and understand drug resistance mechanisms, to date, have primarily focused on cytotoxin export or neutralisation. However, increasing evidence suggests that survival pathways may promote tumour initiation and maintenance. Delineation of the signalling pathways which contribute to disease origin and progression, and the evolution of a drug-resistant phenotype, may allow development of novel pharmacological agents which increase tumour sensitivity to death by conventional cytotoxic drugs. This article reviews current understanding of the contribution of intracellular signalling pathways to survival and neoplasia and how disturbances in a cell's natural ability to initiate the death process limits the killing potential of chemotherapeutic regimes.

Ras

Ras, a G-protein located at the plasma membrane, cycles between a GTP-bound active and a GDP-bound inactive state, in response to growth factor or cytokine interaction with cell surface receptor.[8] Receptor-activated Ras relays extracellular signals through signal transduction pathways, mediating multiple intracellular responses including growth, survival, apoptosis and regulation of the immune response.[9–11] Ras signalling and conversion between its GTP- and GDP-bound forms is a tightly regulated event, orchestrated primarily by guanine-nucleotide exchange factors (GEFs)[12] and GTPase-activating proteins (GAPs),[13] positive and negative regulators of Ras, respectively. Ras is one of the most frequently deregulated proteins in leukaemias, estimated to be mutated in at least 30% of cases.[14] In actuality, Ras deregulation is likely to be significantly higher, as deregulation of GEFs or GAPs, overexpression of growth factor receptors,[15] autonomous cytokine production, such as interleukin-1β,[16,17] mutation

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within the Ras promoter[18] or activation by co-operating oncogenes, such as bcr-abl,[19] may all lead to constitutive signalling of Ras, without mutations in Ras itself. Interestingly, Ras-GAP is localised to chromosome 5 which is frequently deleted in many leukaemias.[20] Moreover, as activated Ras is locked into a GTP-bound state, the requirement for growth factors to initiate Ras signalling cascades is presumably circumvented. Oncogenic Ras signalling influences tumorigenesis, not only due to increased proliferation, but also due to the increased ability of Ras to suppress apoptosis.[9,11] There remains much controversy as to the precise significance of Ras in leukaemia. The high incidence of Ras mutations in the pre-leukaemic disorder, myelodysplasia, suggests that Ras mutations are an initiating event in leukaemias.[21] Conversely, other reports argue that Ras mutations accumulate only with leukaemic progression.[22] Alternatively, in patients lacking Ras mutations, elevated Ras expression is common, resulting in overactive Ras signalling.[23] Recently, an essential role for oncogenic Ras has been reported for tumour maintenance *in vivo*.[24] Despite extensive debate, Ras deregulation is accepted as contributing to disease aggression, immune evasion and reduced patient survival.[11] Patients with N-Ras mutations have a poor response to chemotherapy and low remission rates.[25]

Ras and Bcl-2

Ras is reported to up-regulate expression of Bcl-2 and Bcl-XL in an IL-3-dependent haemopoietic cell line.[26] Bcl-2 and Bcl-XL are members of an extended family of apoptotic regulators, distinguished by their ability to protect cells from a multitude of apoptosis inducing stimuli.[27] Bcl-2 and Bcl-XL are frequently overexpressed in many malignancies, correlating with disease aggression, resistance to chemotherapy and reduced patient survival rates.[28,29] Bcl-2 up-regulation is possibly one of the major mechanisms exploited by Ras to promote survival, and to increase cellular resistance to cytotoxic drug-induced apoptosis.

Ras also lies at the pinnacle of many separate signal transduction pathways[9,11] (Figure 1). Modifications in Ras signalling would alter each of these downstream kinase cascades, permitting amplification of deregulated signals and potentially inducing oncogenesis.

Ras and Bcr-Abl

The bcr-abl oncogene is generated by the translocation of the c-abl tyrosine kinase gene on chromosome 9 into the bcr gene on chromosome 22. Alternative forms of this chimeric protein are the hallmark of chronic myeloid leukaemia (p210) and acute lymphoblastic leukaemia (p190). Bcr-Abl-positive leukaemias are aggressive and drug-resistant, transformed cells being growth factor independent and resistant to apoptosis-inducing stimuli.[30–32] Interestingly, although Ras aberrations are infrequent in Bcr-Abl-transformed cells,[33] increased levels of active GTP-bound Ras are common, presumably due to direct activation of Ras by Bcr-Abl.[19] Thus, it is likely that Ras signalling considerably contributes to disease aggression and resistance to chemotherapeutic drug-induced apoptosis by recruiting multiple signalling cascades to transduce its oncogenic signals. Furthermore, the downstream effector of Ras, PI3-kinase, is also activated by interaction with Bcr-Abl and is required for Bcr-Abl-mediated leukaemogenesis.[34]

Ras and farnesyltransferase inhibitors

The importance of Ras deregulation in leukaemia and other malignancies is reflected in the development of anti-Ras drugs. Ras is synthesised as an inactive precursor molecule, requiring multiple post-translational modifications prior to achieving biologically active status as a mature protein. The most important of these modifications is prenylation of the precursor Ras protein, catalysed by the enzyme, farnesyltransferase.[35,36] The three forms of mutated Ras proteins, N-Ras (commonly associated with haematopoietic neoplasms), Kirsten-Ras (predominantly expressed in pancreatic, colorectal and lung cancer with a prevalence of up to 90%), and Harvey-Ras normally exist as farnesylated proteins *in vivo*.[14,21,35] Inhibition of the farnesyltransferase enzyme prevents post-translational processing of Ras proteins in a highly selective manner. As pharmaceutical agents, farnesyltransferase inhibitors (FTIs) block Ras processing, signalling and transformation in tumour cell lines without overt toxicity. To date, results are extremely promising showing additive, sometimes synergistic growth suppression of cancer cells in culture and in animal studies when inhibitors are used in combination with chemotherapeutic agents. A number of FTIs are currently entering phase II clinical trials.[35,37]

Intracellular expression of an anti-Ras neutralising antibody has been shown to promote apoptosis and tumour regression in Ras-transformed but not untransformed cells.[38] Furthermore, the human reovirus requires an activated Ras signalling pathway to infect cultured cells and has been shown to induce tumour regression in mice.[39]

The ERK pathway

Ras activation leads to stimulation of the serine/threonine kinase, Raf (MAP-KKK), which successively phosphorylates and activates the dual specificity protein kinase, MAP-kinase kinase (MEK), and the extracellular signal related kinases, ERK1 and ERK2. Once activated, ERK1 and ERK2 translocate to the nucleus, where they phosphorylate transcription factors such as c-Fos and ELK1, regulating immediate–early gene expression.[40] While an essential role for ERK has been established in proliferation, its importance with regard to survival remains somewhat less conclusive. There are reports of ERK-mediated protection against apoptosis following growth factor withdrawal.[41] Signalling cascades capable of enhancing cellular survival could potentially increase resistance to cytotoxic drug-induced apoptosis. However, the contribution of ERK to survival under certain circumstances, was not found to extend to protection from cytotoxin-mediated apoptosis.[42] Interestingly, it is the upstream components of this pathway which appear to have an increased capacity for promoting survival. As previously mentioned, oncogenic Ras up-regulates expression of the anti-apoptotic molecules Bcl-2 and Bcl-XL.[26] Activated Raf can suppress apoptosis in an IL-3-dependent cell line following growth factor withdrawal,[43] possibly mediated by interaction with Bcl-2.[44] Raf is also thought to be functionally regulated by PKB/Akt and PKC and to play a role in the phosphorylation of the pro-apoptotic Bcl-2 family member, Bad.[45–47]

PI3-kinase, PKB/Akt and Bad

A prolific quantity of data have established PI3-kinase as an important mediator of survival signals, protecting from mul-

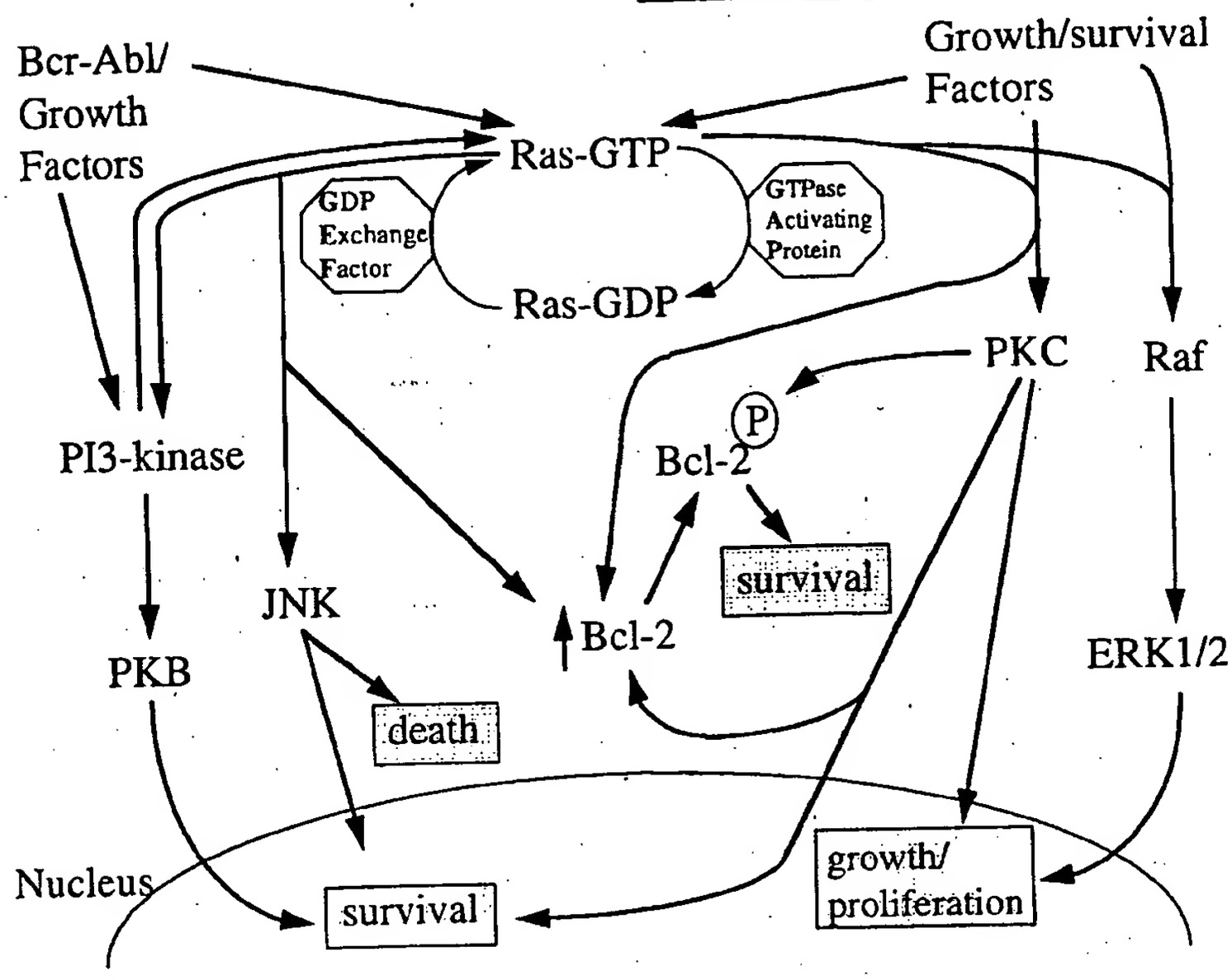

Figure 1 Signalling pathways to and from Ras. Ras deregulation may arise following autocrine growth factor production, Bcr-Abl stimulation, point mutation or dysfunction in the Ras regulators, GEFs and GAPs. Ras mediates growth and survival signals through downstream kinases PI3-kinase/PKB, JNK, PKC and ERK1/2. Ras, PKC and survival pathways up-regulate Bcl-2 expression. PKC also induces Bcl-2 phosphorylation, enhancing its survival promoting capabilities.

tiple apoptosis-inducing stimuli, including growth factor withdrawal and anoikis.[48,49] PI3-kinase may be stimulated by binding of growth or survival factors to cell surface receptors or by interaction with activated Ras.[50] Interestingly, binding of PI3-kinase to activated Ras leads to a further increase in levels of activated Ras.[51,52] Activation of PI3-kinase results in the formation of phospholipids, such as phosphatidylinositol triphosphate (PIP3). PIP3 binds to PKB (also known as Akt), the major downstream mediator of PI3-kinase survival signals, instigating translocation to the plasma membrane, where PKB is phosphorylated and activated by another PIP3-activated kinase, PDK1 (PIP3-dependent kinase 1).[53] The PKB family is now known to consist of three closely related proteins, PKBα, PKBβ and PKBγ.[54] Activated PKB then phosphorylates the pro-apoptotic Bcl-2 family member, Bad.[55] When phosphorylated, Bad is sequestered by the cytoplasmic protein 14–3-3 and apoptosis is inhibited. Conversely, PKB inhibition leads to Bad dephosphorylation and its release from 14–3-3. Bad is now free to bind and neutralise anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL, promoting the induction of apoptosis.[55] The regulation of Bad is becoming increasingly complex with reports that Raf,[46] MAP kinase kinase (MEK),[56] cyclic-AMP-dependent protein kinase (PKA)[57] and the calcium-activated protein phosphatase, calcineurin[58] may all be involved in managing its phosphorylation status. As the functions of PI3-kinase become further elucidated, it is increasingly apparent that signalling alterations can modify multiple downstream effectors, possibly contributing to oncogenesis.

It is likely that many of the effects of deregulated Ras, including chemoresistance, are mediated by PI3-kinase following direct activation by Ras.[50] A study investigating this possibility demonstrated that inhibition of PI3-kinase survival signals, with concomitant inhibition of PKB, in the acute myeloid leukaemia (AML)-derived cell line, HL60, which endogenously expresses an activated N-Ras mutation, greatly increased sensitivity to cytotoxic drug-induced apoptosis.[42] Similar findings were reported by a second group, where PI3-kinase inhibition accelerated daunorubicin-induced apoptosis in the monocytic cell line, U937.[59] Deregulated PKB is evident in a variety of tumours, due to PKB overexpression, constitutive activation by oncogenic Ras, or following inactivation of the tumour suppressor, PTEN.[53,60]

It is known that the survival effects of PI3-kinase are mediated through PKB, but until recently the most significant downstream target of PKB was considered to be Bad, linking cytokine-mediated survival with the Bcl-2 family of apoptotic regulators. However, it seemed unlikely that such an important survival pathway should act through a single downstream effector, especially considering that Bad is poorly expressed in haemopoietic and lymphoid tissue as compared to many normal human tissues and other tumour cell lines.[61] Recently, it has become increasingly evident that Bad does not mediate all the effects of PI3-kinase/PKB. The capacity of particular cytokines to promote survival does not always correlate with PKB and Bad phosphorylation. In fact, the relationship between PKB, Bad and survival is cytokine and cell-type specific.[56,62] Furthermore, Bad does not appear to participate in PI3-kinase-mediated resistance to chemotherapeutic drug-induced apoptosis. PI3-kinase inhibition sensitises myeloid leukaemia cells to drug-induced apoptosis, independently of alterations in Bad phosphorylation status or binding to anti-apoptotic Bcl-2 family members.[42] Recently, additional targets of PKB have been described, which presumably, in time, will be confirmed as participants in PI3-kinase/PKB-mediated

drug resistance.

PKB and transcription factors

NF-κB: NF-κB is a member of the ubiquitously expressed family of Rel-related transcription factors, originally thought to co-ordinate cellular response to infection, stress and injury. However, NF-κB is becoming increasingly recognised as an important contributor to cell survival and protection from apoptosis.[63,64] The mechanisms by which NF-κB can regulate such diametrically opposing events continues to be somewhat enigmatic. NF-κB exists as hetero- and homo-dimers regulated by the inhibitory molecule, IκB (inhibitor of NF-κB). Binding of NF-κB to IκB results in cytoplasmic retention of inactive NF-κB. Dissociation of the NF-κB/IκB complex is initiated by site-specific phosphorylation of IκB by upstream IκB-kinases, IKK1 and IKK2. Phosphorylation of IκB targets it for selective ubiquination and subsequent degradation by proteosomal action. Thus, NF-κB is free to translocate to the nucleus and initiate transcription of target genes. IKK1 and IKK2 are themselves phosphorylated and activated by upstream kinases, most likely members of the extended mitogen-activated protein kinase kinase kinase (MAPKKK) family, such as NIK (NF-κB-inducing kinase) and MEKK1. NIK and MEKK1 are differentially activated by discrete stimuli, leading to selective IKK1 and IKK2 activation and co-ordinated regulation of NF-κB. Differential responses are also achieved through the distinct affinities the NF-κB complex demonstrates for DNA binding sites present in the promoters of target genes. This potentially explains the relationship of NF-κB with seemingly opposing biological events.[63–65]

The intrigue surrounding NF-κB concerns its ability to act as a convergence point for a variety of stimuli, including interleukin-1β, TNFα, UV and survival factors, IGF-1 and PDGF.[63,64,66] Evidence, to date, does not conclusively establish whether NF-κB normally functions as a pro- or as an anti-apoptotic factor. NF-κB protects from apoptosis through transcription of cytokines, including IL-2, IL-6, IL-8, G-CSF and GM-CSF,[64] the Bcl-2 homologue A1,[67] Bcl-x[68] and the inhibitor of apoptosis (IAP) family.[69] NF-κB activation protects from TNFα, ionising radiation and cytotoxic drug-induced apoptosis.[67,70,71] In contrast, NF-κB also aids induction of apoptosis following serum starvation, where dominant negative NF-κB has a protective effect similar to that of Bcl-2 overexpression.[72] NF-κB is required for Fas ligand up-regulation following treatment with DNA damaging agents[73] and transcribes the cell cycle regulators, p53 and c-myc, both of which have been implicated in apoptosis.[64,74] NF-κB-mediated induction of cell cycle arrest may function to provide time for cellular assessment of all incoming signals before determining the fate of the cell. Thus, NF-κB activation can have contrasting cellular outcomes depending on the nature of the external signal received.

Recently, an alternative pathway for NF-κB regulation was described. PKB associates with and activates IKK, inducing phosphorylation and subsequent targeting of IκB for proteosomal degradation in response to PDGF[66] and TNF activation.[75] Thus, NF-κB may possibly be the mechanism by which PI3-kinase/PKB promotes resistance to cytotoxic drug-mediated apoptosis. Inhibition of PI3-kinase would prevent IκB phosphorylation and degradation, ensuring NF-κB retention in the cytoplasm and negation of its protective effects, such as protection from chemotherapy-induced apoptosis.[67,71] Suppression of the caspases (a family of proteases activated during apoptosis)[76] is associated with a drug-resistant phenotype, possibly by NF-κB-mediated IAP expression, which binds and inhibits caspase action.[77,78] NF-κB expression is associated with breast cancer, and may represent a novel therapeutic target in the treatment of cancers with dysfunctional NF-κB.[79]

CREB: The nuclear factor CREB (cAMP-responsive element-binding protein), a component of normal cell cycle machinery is also required for cell survival in certain cell types.[80,81] Phosphorylation of CREB by protein kinase A (PKA) promotes binding to the co-activator CBP (CREB-binding protein) and formation of the CREB/CBP transcription complex.[81,82] It has recently been shown that PKB can also phosphorylate CREB following serum stimulation, inducing CREB/CBP complex formation, translocation to the nucleus and transcription of target genes.[83] It is this pathway which is reported to up-regulate expression of the anti-apoptotic protein and Bcl-2 family member, Mcl-1.[84] Mcl-1 is primarily expressed in haematopoietic cells and plays an important role in cytokine-induced viability.[85,86] Thus, PI3-kinase/PKB-mediated CREB activation may also contribute to resistance against cytotoxin-induced death.

Forkhead family: Insulin receptor-like signalling in *C. elegans* requires PKB homologue activity to suppress the function of DAF-16, a Forkhead family member.[87] A similar pathway has been delineated in mammalian cells, where PI3-kinase/PKB-mediated phosphorylation of Forkhead transcription factors prevents nuclear translocation by inducing binding to the cytoplasmic protein, 14–3-3. Identified members of this family, regulated by PKB, include FKHRL1,[88] FKHR[89] and AFX.[90] Cytoplasmic retention of these transcription factors is thought to prevent the expression of important death inducing genes, such as Fas ligand.[88]

The potential influence of transcription factors on resistance to cytotoxic drug-induced apoptosis is enormous. PKB signalling regulates a number of different transcription factor families, both positively and negatively. Therefore, inhibition of such a critical mediator would have serious repercussions on downstream mediators, determining the final cellular outcome towards life or death.

PKB and caspases

The cysteine proteases, or caspases, are activated following apoptotic insult, destroying and inactivating a multitude of important survival mediators, including the signalling proteins, PKB, Raf-1 and Ras-GAP,[91] the DNA repair-associated enzyme, PARP, and components of the cytoskeleton, including actin, lamin and fodrin.[92] Caspases possess autocatalytic activity and can convert downstream caspases from inactive procaspase zymogen to active caspase, amplifying the death cascade.[76] Moreover, caspase-mediated cleavage of Bcl-2 results in its conversion from an anti-apoptotic to a pro-apoptotic protein.[93] The importance of caspase signalling in apoptosis is further highlighted by a recent report, where chemoresistance in the leukaemic cell line, TUR, was attributed to defects in caspase activation.[77]

Procaspase 9 is yet another target for PKB activity. PKB-mediated site-specific phosphorylation of procaspase 9 results in defective proteolytic processing of this initiator caspase.[94] Inhibition of PI3-kinase and PKB eliminates this block in pro-

caspase 9 processing. As caspases are customarily activated following cytotoxic drug treatment, such treatment in combination with PKB inhibition may lead to synergistic activation of the caspases and potentiation of apoptosis. Indeed, PI3-kinase inhibition enhances processing of caspase 3 in TNF- and Fas-treated, but not untreated cells.[95]

PKB and glycogen synthase kinase 3

Glycogen synthase kinase 3 (GSK3), is also a substrate for PKB action and the first to be identified *in vivo*.[96] GSK3 phosphorylates glycogen synthase (GS), regulating glycogen synthesis in mammals. Cellular stimulation with insulin or IGF-1 leads to PKB-mediated phosphorylation of GSK3. GSK3 is inactivated by phosphorylation, permitting glycogen synthesis by the no longer inhibited GS.[97] GSK3 is also involved in regulation of a range of other substrates including metabolic enzymes, and transcription factors, CREB, c-Myc and AP1, the regulatory subunit of cyclic-AMP-dependent protein kinase and the translation factor eIF-2B.[96,98,99] The importance of GSK3 in chemoresistance has not yet been established. However, considering its regulation by the survival promoting molecule PKB, the role played by GSK3 warrants further investigation.

Following reports establishing PI3-kinase as a contributor of consequence to cytotoxic drug resistance in leukaemia cell lines, most likely through PKB signalling,[42,59] we have speculated upon the potential contribution of known PKB substrates to this process (Figure 2). The importance of PKB signalling to survival is underlined by the finding that PKB is also cleaved and inactivated by caspase action during apoptosis.[91] Recently, new targets of PKB signalling have been established including nitric oxide[100,101] and presumably more remain to be elucidated. Identification of the specific components of the biochemical pathway, which individually or in combination, mediate PI3-kinase resistance to cytotoxic drug-induced apoptosis, will be an exciting challenge for future forthcoming research.

Protein kinase C (PKC)

The PKC family of serine/threonine protein kinases play an important, yet diverse role in signal transduction, mediating the intracellular effects of many extracellular stimuli including growth factors, hormones and drugs. In responding to these multiple stimuli, PKC activity influences mitogenesis, differentiation, survival and apoptosis.[102–104] The multiplicity of roles in which PKC is implicated presumably arises from the 11 isoenzymes of which the family is composed.[105] These can be categorised into three groups, the classical or calcium-dependent isoenzymes (PKC α, βI, βII and γ), the novel or non-calcium-dependent (PKC δ, ϵ, η, θ and μ) and the atypical subgroups (PKC ζ, ι or λ). Each of these subgroups possess structural differences, reflecting differential requirements for cofactors essential for activation. Both the calcium and non-calcium-dependent subgroups require the second messenger diacylglycerol (DAG) as well as membrane-associated phospholipids for activation, while the atypical isoforms are independent of both calcium and DAG. The versatility of PKC action is primarily thought to be due to its activation by DAG, produced by intracellular phospholipid turnover.[102–104]

The role of PKC in initiating and/or co-operating in disease progression remains unclear. Downregulation of PKCβ and up-regulation of PKCϵ expression are early events in prostate

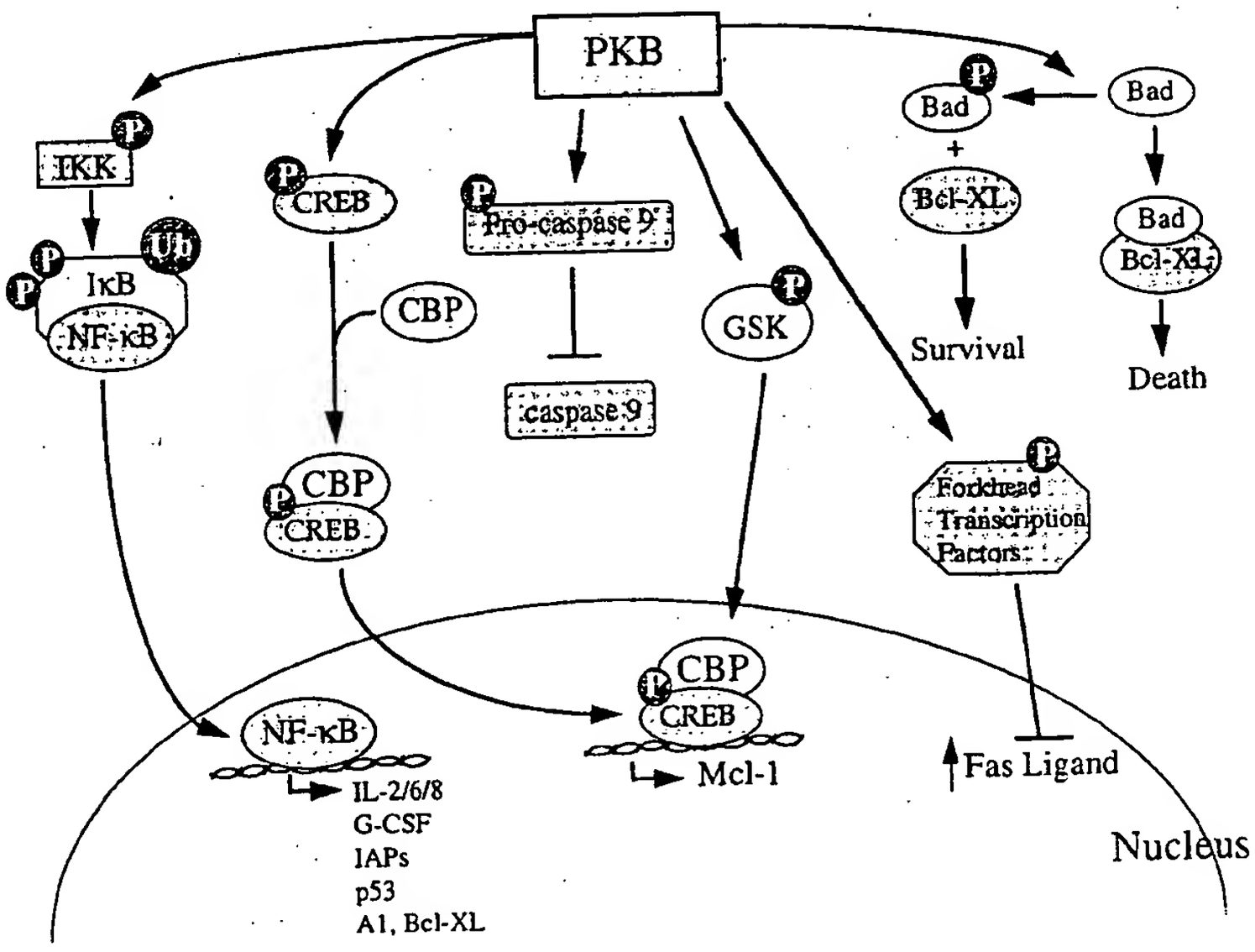

Figure 2 PKB mediates survival through multiple downstream effectors including the transcription factors, NF-κB, CREB/CBP and the forkhead family. PKB signalling inhibits processing of pro-caspase 9 to its active conformation. PKB phosphorylates the pro-apoptotic Bcl-2 family member Bad, preventing binding and neutralisation of anti-apoptotic family members. PKB also regulates GSK3 activity, influencing downstream effectors such as CREB.

neoplasia, with PKCα and PKCζ also demonstrating enhanced expression.[106] Over-expression of PKCα and PKCβ are associated with a less aggressive breast cancer phenotype.[107] PKCβ has an anti-oncogenic function in colon cancer cells.[108] Conversely, PKCβ over-expression contributes to fibroblast transformation.[109,110] Opposing reports have been published regarding the transforming abilities of PKCα in fibroblasts.[111,112] PKCε is oncogenic in colonic epithelial cells,[113] but although having increased expression in hepatocellular carcinomas, does not appear to be involved in transformation or disease progression.[114] Reports concerning the contribution of individual PKC isoenzymes to oncogenesis are often conflicting, presumably resulting from differential isoenzyme tissue expression, cellular localisation, regulation and function. Increased understanding of the contribution of specific PKC isoenzymes to malignancy would allow targeting of PKC as a potential point of therapeutic intervention. In support of this theory is the recent report that antisense inhibition of PKCα can reverse the neoplastic properties of human lung carcinoma cells.[115]

PKC and multidrug resistance

Increased expression and activity of PKC, in particular PKCα, has been correlated with the development of a multidrug-resistant (MDR) phenotype[116] through PKC-mediated phosphorylation of p-glycoprotein.[117] Indeed, there is a strong correlation between PKC and MDR-gene expression in both primary and relapsed state AML.[118] In haematopoietic cells, inhibition of PKC dramatically increases sensitivity to chemotherapeutic drug-induced apoptosis, involving alterations in the phosphorylation status of the anti-apoptotic protein, Bcl-2.[119,120] PKC is considered to be the upstream kinase responsible for Bcl-2 phosphorylation, a post-translational modification essential for maximal anti-apoptotic function.[121,122] In addition, PKCε has been reported to induce Bcl-2 expression,[123] associated with disease aggression, chemoresistance and poor clinical prognosis.[28,29]

PKC and other interacting pathways

PKC may also contribute to neoplasia and chemoresistance, through associations with other signalling molecules. Active PKC and Ras co-operate to promote proliferation and transformation,[124,125] however, following PKC inhibition, the pathway stimulated by Ras is apoptosis.[125] Both Ras and PKC enhance Bcl-2 expression levels and anti-apoptotic function.[26,121] PKB may promote survival by mitochondrial targeting of Raf, in a PKC-dependent manner, inducing Bad phosphorylation and promoting survival.[46] p90RSK signalling also blocks Bad-mediated death through a PKC-dependent pathway.[126] Indeed, PKC itself has also been reported to activate Raf.[47] Thus, several of the effects of Ras and Bcl-2 in leukaemia, such as disease aggression and chemoresistance, may be at least partially mediated by PKC, or rely on PKC activity as a co-operating lesion. Furthermore, PKC contributes to Bcr-Abl-mediated drug resistance.[127] Induction of PKC protects from Fas-mediated apoptosis in viral infected T cells.[128] PKCζ is critical for NF-κB activation, by inducing dissociation from IκB.[129] As described earlier, NF-κB exerts a survival signal and may contribute to chemoresistance. Certain PKC isoforms may be regulated by PI3-kinase through PDK-1.[130] Thus, considering the relationship between PKC, MDR, Bcl-2 and multiple signalling molecules, PKC inhibition may potentially be of immense importance in treatment regimens, augmenting the cytotoxicity of chemotherapeutic drugs and potentiating the killing efficacy of death pathways, such as Fas/CD95. The combinatorial activity of PKC and multiple signalling pathways may also significantly influence cell viability, providing yet another point of therapeutic intervention.

Stress-activated protein kinases

The stress-activated protein kinases (SAPK) or c-jun N-terminal kinases (JNK), a subgroup of the mitogen-activated protein (MAP) kinase family, are involved in the cellular response to toxins, physiological stresses, inflammatory cytokines, DNA damage and heat shock.[131,132] Upon receipt of extracellular stress, JNK is phosphorylated and activated, in turn phosphorylating and activating c-Jun, a member of the dimeric transcription factor, AP-1. Other AP-1 components include c-Fos and ATF2. Both ATF2 and c-Fos, in addition to c-Jun are phosphorylated and activated by JNK.[133] c-Fos may also be activated by ERK in response to mitogenic stimulation.[134] A considerable debate regarding the requirement for JNK in the apoptotic cascade is currently being contested. Substantial evidence has implicated JNK and c-Jun as essential components of the apoptotic cascade. Activation of c-Jun is required for apoptosis following NGF withdrawal in neuronal cells[41,135] and following growth factor withdrawal from IL-6- and IL-2-dependent lymphoid cell lines.[136] Recently, JNK activity was found to be a necessary requirement for UV-induced apoptosis in primary murine embryonic fibroblasts, modulating cytochrome-c release from the mitochondria.[137] Conversely, apoptosis proceeds unhindered in c-Fos/c-Jun null mouse embryos, indicating that AP-1 is a non-essential component of the death cascade.[138] Furthermore, thymocytes lacking an upstream component of the JNK pathway, SEK1, are more susceptible to Fas-mediated apoptosis arguing a protective function for JNK.[139] Interestingly, the same SEK1 mutation did not affect apoptosis in embryonic stem cells or T cells.[139] The role of JNK in Fas-mediated apoptosis is also controversial with opposing reports regarding the requirement for JNK. Several studies suggest that JNK is critical for Fas-induced apoptosis, involved in up-regulating Fas ligand in T cells,[140] neuronal cells[141] and epithelial cells.[142] However, substantial evidence suggests that JNK is non-essential for Fas-mediated apoptosis in haemopoietic cell lines.[143,144] Thus, in certain situations, JNK activity is a positive event in the induction of apoptosis, but in alternative situations and cell types, c-Jun inhibits apoptosis and contributes to proliferation or differentiation.[131,132] Therefore, cell type[139] and inducing stimulus[145] appear to determine whether JNK activation is a causal or secondary event in apoptosis.

The final biological outcome mediated by JNK activity appears dependent on the state of activation or inactivation of other signalling pathways within the cell. Moreover, JNK activity may be recessive to survival pathway signalling.[41] JNK, like many other signalling molecules seems to exert many varied and sometimes opposing functions, including transformation, growth and development, death and survival.[131,132] While JNK participation is considered essential for neuronal cell apoptosis,[135] its importance in haematopoietic cells remains less straightforward. Ras activates JNK following IL-3 stimulation,[146] and JNK is essential for Ras[147] and Bcr-Abl-mediated transformation.[148] It is also required for the induction of apoptosis in lymphocytes.[136] Furthermore, JNK

has been associated with chemoresistance, where cells lacking JNK activity are refractory to cytotoxic drug-induced apoptosis.[149,150] However, in sensitive cells, JNK triggers DNA damaging drug-induced apoptosis by activating the caspase cascade.[150] Thus, JNK may determine the clinical response of tumour cells to cytotoxic therapies. The influence of JNK depends not only on cell type[139] and external stimulus,[145] but also on the activation status of other regulatory signalling pathways. Indeed, the chosen route towards life or death frequently depends on the shift in balance between JNK and ERK signalling.[41,151,152] Furthermore, oncogenic Ras is a potent inducer of AP-1.[147] As deregulated AP-1 expression can initiate neoplastic transformation, AP-1 may be a critical Ras effector, contributing to malignancy and chemoresistance.

The Bcl-2 family of apoptotic regulators

The anti-apoptotic protein, Bcl-2, was first recognised for its involvement in the t(14;18) chromosomal translocation, associated with extensive Bcl-2 over-expression in B cell lymphomas.[153] Bcl-2 is also distinguished for its capacity to promote cellular survival rather than proliferation, and to protect against almost all known apoptosis-inducing stimuli, including cytotoxic drugs, growth factor withdrawal, c-myc, radiation, heat and in some cases death receptor activation.[154–159] The related anti-apoptotic Bcl-2 family member, Bcl-XL also confers a drug-resistance phenotype.[160] To date, 17 mammalian homologues of Bcl-2 have been described acting as either anti- (Bcl-XL, Mcl-1, A1, Bcl-w and Boo) or pro-apoptotic proteins (Bax, Bak, Bok, Bik, Blk, Hrk, BNIP3, Bim, Bad, Bid, Bcl-Xs and Diva).[27,161,162] Bcl-XL and Bcl-Xs arise through alternative mRNA splicing from the same gene.[163]

Bcl-2 has been found at inappropriately high levels in half of all human cancers,[164] frequently in the absence of gene rearrangements, including non-Hodgkin's lymphoma,[165] acute leukaemias[28,166] and breast cancer,[167] suggesting a fundamental role for Bcl-2 in carcinogenesis. Bcl-2 is particularly significant in AML, where drug resistance becomes increasingly prevalent with disease progression. Bcl-2 is expressed in over 87% of AML cases at presentation, increasing to 100% at relapse.[168] AML blasts also display autocrine growth factor production, up-regulating Bcl-2 expression and increasing resistance to treatment-induced apoptosis.[169] Thus, Bcl-2 potentiates cell survival, contributes to cancer initiation, correlates with disease aggression and chemoresistance and is overall a poor prognostic marker for successful treatment, patient survival and clinical outcome.[28,168,170–173] Transfection of Bcl-2 or Bcl-XL into cell lines greatly increases resistance to numerous insults, including cytotoxic drug-induced apoptosis.[154,155,160] Conversely, inhibition or abatement of Bcl-2 function using antisense oligonucleotides reverses chemoresistance,[174,175] as does elevation of Bcl-2 antagonists, Bax and Bcl-Xs.[176,177] Furthermore, attenuated Bax expression behaves similarly to increased Bcl-2 expression, augmenting resistance to apoptotic stimuli and enhancing tumour aggression.[178] Thus, the relative balance between pro- and anti-apoptotic molecules appears to determine the overall cellular survival advantage or disadvantage.[179]

Initially, it was suggested that the Bcl-2 family exerted their effects by heterodimerisation, resulting in neutralisation of one of the binding partners.[180] However, recent reports have challenged the dogma of heterodimerisation, determining that there exists both heterodimerisation-dependent and -independent mechanisms for the regulation of apoptosis. Hsu *et al*[181] reported that Bax heterodimerisation was an artefact of non-ionic detergent perturbation. Another report determined that while the Bcl-2/Bax heterodimer could promote apoptosis, protection correlated, not with heterodimerisation levels, but rather with the levels of unbound Bcl-2.[182] The survival mechanism employed by the Bcl-2 homologues, A1 and Bcl-w, only partially correlates with dimerisation.[183] Bcl-XL also regulates apoptosis by heterodimerisation-dependent and -independent mechanisms.[184]

Bcl-2 and signalling pathways

Survival factors have been reported to inhibit apoptosis by maintaining intracellular Bcl-2 expression levels.[185] It is likely that these survival factor-regulated kinase cascades also regulate Bcl-2 phosphorylation status, where PKC-mediated phosphorylation is considered essential for optimum anti-apoptotic activity.[121,122] Phosphorylation may also influence interactions between the Bcl-2 family and targets such as the apoptosis activating factor, Apaf-1. Bcl-XL binding to Apaf-1 is thought to inhibit pro-caspase 9 processing.[186] However, a recent publication has disputed this theory, having found no evidence of interaction between anti-apoptotic family members and Apaf-1.[187] Bcl-2 family members have also been suggested to act at the level of the mitochondria regulating release of the apoptosis promoting factor, cytochrome c, required for Apaf-1 processing of pro-caspase 9.[188,189] Bcl-2 functionally cooperates with Raf to suppress apoptosis, possibly by targeting Raf to the mitochondria, inducing subsequent Bad phosphorylation and inhibition of apoptosis.[44,46]

Bcl-2 and p53

Elevated Bcl-2 expression may also be related to loss of p53 function, prevalent in over 50% of cancers.[190,191] p53 directs cell cycle arrest following DNA damage, and initiates DNA repair when possible prior to further replication. However, following excessive DNA damage, apoptosis is promoted.[192,193] Functional p53 not only acts as a repressor of Bcl-2 gene expression, but also promotes transactivation of Bax.[194] Thus, loss of p53 not only disables a cells intrinsic mechanism to survey damage, but may also dramatically alter the anti- to pro-apoptotic ratio of Bcl-2 family proteins.

Although the mechanisms of action of Bcl-2 family members remain to be clarified, their anti- and pro-apoptotic capabilities remain undisputed and are unlikely to arise from a single function, but rather a composite of those previously mentioned. Interference with Bcl-2 function or elevation of Bcl-2 antagonist levels, removes an important barrier for the successful progression of apoptosis, and efficient induction of chemotherapeutic drug-induced death. However, attempts to predict clinical outcome based on Bcl-2 studies alone may not be accurate, as individual malignancies exhibit aberrations in specific Bcl-2 family members, influencing chemoresistance and final disease prognosis.

Fas/APO-1/CD95

The cell surface death receptor, Fas/APO-1/CD95 transduces apoptotic signals upon activation by specific death ligands, such as agonistic Fas antibody or under physiological conditions by interaction with cognate Fas ligand.[195] Fas is a

member of the extended tumour necrosis factor (TNF) superfamily of death receptors and plays an important role in normal physiology.[195] The binding of cognate interacting ligands induces oligomerisation of Fas receptor and the recruitment of signalling molecules, Fas-associated death domain (FADD) and pro-caspase 8 to its cytoplasmic death domains, which together are known as the death-inducing signalling complex or DISC.[195] Initiation of the caspase cascade results in subsequent cell death by either mitochondrial or non-mitochondrial pathways.[159] Bcl-2 can inhibit mitochondrial apoptosis downstream of the DISC.[196]

The significance of the Fas death system in chemotherapeutic drug-induced apoptosis is currently the subject of conflicting discussion. Chemotherapeutic drugs have been reported to up-regulate Fas ligand expression on Fas-expressing leukaemia cells.[197] Apoptosis is then triggered by subsequent binding of Fas ligand to Fas receptor. Similar scenarios have also evolved in non-leukaemic tumours, including hepatoblastoma and neuroblastoma.[198–200] Chemotherapeutic drugs have also been reported to increase sensitivity to Fas-induced apoptosis in both pre-B ALL cells[201] and prostate tumour cells.[202] If Fas involvement in chemotherapy consistently described a successful anti-tumour therapy, it would have far reaching therapeutic consequences. However, publications on Fas involvement in tumours have also illustrated many opposing theories.

While drug-induced up-regulation of Fas on tumour cells would no doubt enhance sensitivity to FasL-positive T cells and NK cells, similar up-regulation of FasL would allow immune evasion, making the tumour immune-privileged and having an injurious effect on the immune system.[195,203] Indeed, expression of FasL by tumour cells is often accompanied by loss of Fas, thus eliminating the possibility of self-induced apoptosis.[204] There is substantial evidence in support of the viewpoint that cytotoxic drug-induced apoptosis is independent of Fas/Fas ligand recruitment, where blocking the Fas receptor with neutralising antibody fails to inhibit chemotherapeutic drug-induced apoptosis.[205,206] In acute lymphatic leukaemia cells, although drug-induced apoptosis is associated with increased Fas ligand expression, death appears independent of Fas signalling.[207] Furthermore, analysis of AML patients found no notable differences in Fas expression between patients who achieved complete remission and patients who developed refractory or relapsed AML.[208] Interestingly, Micheau *et al*[209] report that anti-cancer drugs induce apoptosis by activating the Fas receptor and inducing interaction with FADD, but in a FasL-independent manner. This report may resolve some of the arguments surrounding the necessity of Fas ligand for drug-induced apoptosis. Moreover, in cells expressing Fas antigen, there exists both Fas-dependent and -independent mechanisms of drug-induced apoptosis.[210] It is likely that other intracellular factors such as signalling pathways and anti-apoptotic molecules play an important role in determining final sensitivity to Fas-induced death. An undisputed finding is that synergy between anti-Fas and cytotoxic drug treatment is evident in multiple cell lines, indicating that these apparently distinct signalling pathways may share a common convergence point downstream in the apoptotic cascade.[205,211–213]

Fas and p53

Fas/FasL up-regulation and sensitivity to Fas-induced death may depend on the presence of functional p53.[198] This observation may explain the non-participation of the Fas system in studies where the cell lines employed were p53 null or p53 negligible.[205] Lack of functional p53 may explain cellular inability to up-regulate Fas and FasL following drug treatment, indicating that there exists alternative pathways by which chemotherapeutic drugs mediate their cytotoxic effects.

Fas and survival signals

The relationships between the instructive death signal from Fas and survival signals are incompletely elucidated, but most studies, to date, indicate that survival pathways negatively regulate Fas-induced apoptosis. Oncogenic Ras suppresses Fas-mediated apoptosis by down-regulating Fas expression.[214] Indeed, Ras transformed cells have enhanced resistance to Fas-induced death.[215] Fas-induced apoptosis is counteracted by PI3-kinase signalling, in a PKB-dependent manner.[216] The tumour suppressor and PKB inactivator, PTEN, was found to sensitise glioma cells to Fas- but not chemotherapeutic drug-induced apoptosis.[217] Inhibition of PKC also promotes sensitivity to Fas-mediated apoptosis.[218]

Thus, sensitivity to Fas-induced apoptosis may be dependent on the state of activation of intracellular survival and death pathways, where Fas-mediated death may be potentiated by inhibition of survival pathways such as PI3-kinase,[216] or activation of a death pathway, such as JNK.[202] Such signal-specific targeting may prove to be of consequence in increasing sensitivity to both Fas- and cytotoxin-mediated apoptosis, with potentially immense implications for combination therapies.

Discussion

Drug resistance has previously been attributed to modifications in drug transport, metabolism and cellular repair. It is now increasingly evident that exploitation of survival pathways also significantly contributes to chemoresistance. Indeed, haematopoietic cytokines inhibit apoptosis induced by a range of cytotoxins,[219] presumably by activation of downstream survival pathways. Therefore, drug resistance appears to be multi-factorial, where several anti-apoptotic mechanisms are recruited to equip the cell with an increased survival capacity in order to facilitate disease progression and evade drug-induced apoptosis.[220,221]

The development of oncogenic lesions, where the capacity to promote apoptosis but not proliferation is abrogated, must be accompanied by anti-apoptotic aberrations in order to achieve malignancy. Bcl-2 inhibits apoptotic death normally induced by c-myc[222] and Ras co-operates with c-myc and p53 in cellular transformation.[223] Thus, secondary lesions or rescuing survival signals are essential to dissuade genetically unstable cells from automatically undergoing apoptosis. As such, therapies selective for Ras, Bcl-2 and other survival signals should be relatively specific for transformed cells.

Tumours possessing defects in their apoptotic pathway, may be considered, not so much drug resistant, as inherently resistant to death. Such defects are potentially more lethal than mechanisms which have developed in response to particular groups of cytotoxic agents, such as P-glycoprotein. As Ras dysfunction is a common activating lesion in leukaemias and other neoplasms, it is possible that Ras frequently provides the co-operating anti-apoptotic lesion, limiting the ability of cytotoxic drugs to engage cell death. Inhibitors of survival

pathways would therefore significantly influence the cellular decision regarding life or death in the presence of chemotherapeutic drugs. In the absence of their obligatory survival signal, cells are pushed towards apoptosis with undoubtedly crucial consequences for chemotherapy (Figure 3).

The clinical potential for protein kinase inhibitors is being increasingly recognised. Two therapies specific for Ras-transformed malignant cells include farnesyltransferase inhibitors and reoviral infection. These are currently showing promising results with low toxicity in clinical and preclinical trials, respectively.[35,39] The c-Abl tyrosine kinase inhibitor, STI571, has been shown to inhibit Bcr-Abl-mediated transformation both *in vitro* and *in vivo* and is currently undergoing phase I clinical trials.[224,225] PKC inhibitors under clinical evaluation include UCN-01 and PKC412.[226,227] The PKC inhibitor LY333531 specifically targets PKC β and has successfully demonstrated anti-tumorigenic activity in preclinical trials.[228] Although Bcl-2 antisense treatment has demonstrated significant anti-apoptotic effects,[229] the lack of a specific biochemical inhibitor is limiting clinical investigation. Likewise, no specific inhibitor of either PKB or JNK has been identified to date. A role for PI3-kinase in chemoresistance *in vitro* has only recently been reported[42,59] and the potential (and toxicity) of the PI3-kinase inhibitors LY294002 and wortmannin have not yet been clinically evaluated.

Survival signals are unique in protecting cells by preventing the conversion of cytotoxin-induced injury into death signals. Survival signals, as their name suggests, increase the capacity of a cell to survive insult, possibly by allowing time for cell repair or the accumulation of anti-apoptotic lesions, thus promoting disease aggression and chemoresistance. An increased understanding of the molecular defects leading to overactive survival pathways, or suppression of apoptosis, could translate into a rational approach for the development of selective anticancer therapies, custom-made for individual malignancies. Specific inhibition of survival signals could reverse the intracellular decision towards survival, leading to increased apoptosis and tumour regression. An increased understanding of the tissue-specific requirements for survival and potential exploitation of cell-type specific pathways may yield new and more selective combination therapies, such as are already proving effective in the treatment of IL-6-dependent multiple myeloma.[230]

In conclusion, despite considerable efforts, the problem of drug resistance remains largely unresolved. The significance of survival pathway signalling and co-operation with anti-apoptotic processes, in addition to their inhibitory influence over death pathways and promoters of apoptosis, require better understanding before scientific progress may be converted into clinical advances.

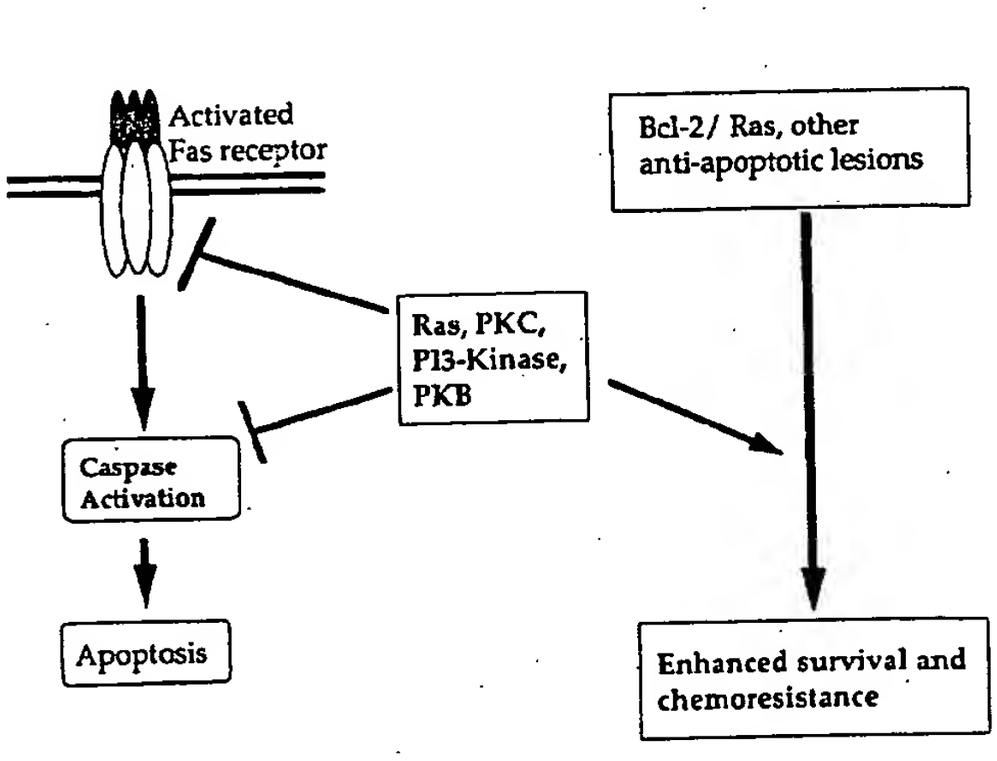

Figure 3 The survival molecules Ras, PI3-kinase, PKB and PKC influence survival by at least two mechanisms: (1) by inhibiting the Fas-induced death cascade, and caspase activation; and (2) by co-operating with anti-apoptotic lesions such as Bcl-2. Both mechanisms limit the induction of apoptosis, enhance survival and contribute to chemoresistance.

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Abstract
Drug resistance, to date, has primarily been attributed to increased drug export or detoxification mechanisms. Despite correlations between drug export and drug resistance, it is increasingly apparent that such mechanisms cannot fully account for **chemoresistance** in neoplasia. It is now widely accepted that chemotherapeutic drugs kill tumour cells by inducing apoptosis, a genetically regulated cell death programme. Evidence is emerging that the exploitation of survival pathways, which may have contributed to disease development in the first instance, may also be important in the development of the **chemoresistance**. This review discusses the components of and associations between multiple **signal**ling cascades and their possible contribution to the development of neoplasia and the chemoresistant phenotype. [References: 230]

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